# AN INVESTIGATION INTO THE PATHOGENESIS OF RAYNAUD'S DISEASE: THE ROLE OF THE VASCULAR ENDOTHELIUM

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## Declaration

I declare that this thesis was written entirely by me and represents all my own work, except for the procedures listed below and acknowledged in the text.

1. Gluteal fat biopsies were performed by Dr Charles J. Ferro of the Clinical Pharmacolgy Unit and Research Centre, University of Edinburgh, Western General Hospital.

2. Scanning and transmission electron microscopy was performed by Dr Kathy P.B. Cracknell and colleagues at the Division of Physiology, St Thomas' Hospital, London.

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## Associated Publications

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## Abstract

Raynaud's disease, first described in 1862, is characterised by intense vasospasm of the extremities, particularly the digits, in response to cold exposure or marked emotion. It is a common condition, occuring in up to 20% of the population, and more often in females. Currently, neither the mechanism of cold-induced vasoconstriction, nor the pathogenesis of Raynaud's disease, is fully understood. The vascular endothelium is known to generate several potent vasoactive substances including the vasoconstrictor endothelin (ET), and the vasodilators, nitric oxide (NO) and prostacyclin ($PGI_2$). A temperature-dependent disorder of the endothelium, involving either overproduction of a vasoconstrictor or underproduction of a vasodilator, might be a critical factor underlying the pathogenesis of Raynaud's disease. The aim of this thesis was to investigate the role of the vascular endothelium in the development of cold-induced vasoconstriction, and in the pathophysiology of vasospasm in Raynaud's disease.

Experiments were performed on resistance arteries *in vitro* and *in vivo*. The small vessel arteriograph (perfusion myograph) was used to study isolated arteries *in vitro*. Cooling rat mesenteric resistance arteries from 37°C to 24°C was found to increase the senstivity to ET-1 and the $\alpha_1$-adrenoceptor agonist, phenylephrine (PE). The endothelium was partly responsible for mediating the cold-induced potentiation of the contractile response to these agents. Maximal contraction to KCl was reduced during cooling, but was restored after removal of the endothelium, indicating that endothelium-dependent dilators inhibit the contractile response to KCl at 24°C. Cooling did not significantly effect the sensitivity of the arteries to the endothelium-dependent and -independent dilators, acetylcholine (ACh) and sodium nitroprusside (SNP) respectively, although there was a tendency for the sensitivity to be reduced, suggesting NO-synthase and vascular smooth muscle sensitivity to NO is potentially depressed during cooling. Having determined the physiological effect of cooling on the response to various agents, and the role of the endothelium, studies were repeated

in resistance arteries dissected from gluteal fat biopsies taken from control subjects and Raynaud's patients, in order to investigate the pathophysiology of cold-induced vasospasm. The results from these studies revealed that arteries from patients with Raynaud's disease had an enhanced sensitivity to ET-1 compared to controls at 37°C. A possible explanation for this could be reduced dilator function in Raynaud's patients, as suggested by the responses to ACh and SNP, which were both attenuated in vessels from patients with Raynaud's disease compared to controls. This implied that there was a decrease in NO-synthase activity and smooth muscle sensitivity to NO at 37°C. Interestingly, at 24°C, the responses to ET-1, ACh and SNP were similar in control arteries and in those from patients with Raynaud's disease. It would appear from the ACh data that NO-synthase activity was enhanced in vessels from Raynaud's patients during cooling, with the increase in NO production opposing the contraction to ET-1. This may, of course, reflect only agonist-stimulated NO generation, whilst endogenous production may be depressed.

Studies *in vivo* used the autoperfused hindlimb of the anaesthetised rat, in which stepped-cooling of the blood entering the hindlimb produced a rise in hindlimb perfusion pressure. Possible mediators of this cold-induced vasoconstriction were identified using antagonists selective for $\alpha$-adrenoceptors and ET-receptors. The $\alpha_1/\alpha_2$-adrenoceptor antagonist, phentolamine, attenuated the cold-induced vasoconstriction, and was the only agent to have an effect that was significant in this model.

The results demonstrate that the endothelium is an important modulator of cold-induced effects on the response to several vasoconstrictor agents, either by enhancing contraction through the release of vasoconstrictors such as ET, or by depressing contraction through the release of dilators, such as NO or $PGI_2$. The results from the gluteal biopsy studies imply that the enhanced sensitivity to ET-1 at 37°C in Raynaud's patients is dependent on changes in dilator function, and not to altered

sensitivity of the vascular smooth muscle to ET-1. Although an increase in the response to ET-1 was not found at 24°C, the results obtained at 37°C would support the hypothesis that the release and/or actions of NO is reduced in Raynaud's disease, allowing enhanced constriction to ET. These results suggest that the vascular endothelium is involved in the pathophysiology of Raynaud's disease.

## List of figures

[Acetylcholine (ACh); endothelin-1 (ET-1); $N^G$-nitro-L-arginine methyl ester (L-NAME); phenylephrine (PE); potassium chloride (KCl); sodium nitroprusside (SNP)].

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## Abbreviations

| | |
|---|---|
| α | alpha |
| A-V | arteriovenous |
| ACE | angiotensin converting enzyme |
| ACh | acetylcholine |
| ADMA | asymmetric dimethyl-L-arginine |
| ANA | anti-nuclear antibodies |
| Ang I | angiotensin I |
| Ang II | angiotensin II |
| ATP | adenosine triphosphate |
| β | beta |
| BK | bradykinin |
| $Ca^{2+}$ | calcium ion |
| cAMP | cyclic adenosine monophosphate |

| | |
|---|---|
| cDNA | complementary DNA |
| cGMP | cyclic guanosine monophosphate |
| DAG | diacylglycerol |
| DNA | deoxyribonucleic acid |
| $EC_{50}$ | the concentration producing 50% of the maximum contraction |
| ECE | endothelin converting enzyme |
| EDHF | endothelium-derived hyperpolarising factor |
| EDRF | endothelium-derived relaxing factor |
| $E_{max}$ | maximal response |
| ET | endothelin |
| $ET_A$ | endothelin-A type receptor |
| $ET_B$ | endothelin-B type receptor |
| $ET_C$ | endothelin-C type receptor |
| G-protein | guanine nucleotide binding regulatory protein |
| $H^+$ | hydrogen ion |
| HLPP | hindlimb perfusion pressure |
| 5-HT | 5-hydroxytryptamine (serotonin) |
| ID | internal diameter |
| $IP_3$ | inositol trisphosphate |
| KCl | potassium chloride |
| L-NAME | $N^G$-nitro-L-arginine methyl ester |
| L-NMMA | $N^G$-monomethyl-L-arginine |
| LD | lumen diameter |
| MAP | mean arterial pressure |
| MCh | methacholine |
| min | minute |
| mmHg | millimeters of mercury |
| n | number |

| | |
|---|---|
| NA | noradrenaline |
| $Na^+$ | sodium ion |
| NEP | neutral endopeptidase |
| NO | nitric oxide |
| NOS | nitric oxide synthase |
| PAF | platelet activating factor |
| PE | phenylephrine |
| $PGE_2$ | prostaglandin $E_2$ |
| $PGG_2$ | prostaglandin $G_2$ |
| $PGH_2$ | prostaglandin $H_2$ |
| $PGI_2$ | prostacyclin |
| PKC | protein kinase C |
| $PLA_2$ | phospholipase $A_2$ |
| PLC | phospholipase C |
| PSS | physiological salt solution |
| SEM | standard error of the mean or scanning electron microscopy |
| SNP | sodium nitroprusside |
| SRTX | sarafotoxin |
| TEM | transmission electron microscopy |
| $TXA_2$ | thromboxane $A_2$ |
| VWF | vibration white finger |
| °C | degree centigrade |
| % | percent |

# CHAPTER 1:
# INTRODUCTION

## A. The Vascular Endothelium

The wall of every blood vessel, except for the capillaries, is made up of three separate layers: the innermost *tunica intima*, which consists of a single continuous layer of endothelial cells; the *tunica media*, made up of smooth muscle cells (absent from capillaries); and the outer *tunica adventitia*, which consists of connective tissue (collagen and elastin) and fibroblasts (Figure 1.1). The vascular endothelium, once thought to act merely as a lining of blood vessels, is now known to play a central role in the regulation of blood flow and vascular tone. In addition to the sympathetic nervous system and circulating hormones, the activity of vascular smooth muscle is controlled by several potent vasoactive substances generated by the endothelium. These substances include the vasodilators, **nitric oxide** (Furchgott & Zawadzki, 1980; Palmer *et al.*, 1987) and **prostacyclin** (Moncada *et al.*, 1976), and the vasoconstrictors, **endothelin** (Yanagisawa *et al.*, 1988), **angiotensin II** (Webb & Cockcroft, 1990) and **thromboxane $A_2$** (Auch-Schwelk *et al.*, 1990).

The following section shall focus primarily on endothelin and nitric oxide, but angiotensin II, thromboxane $A_2$, superoxide anions, prostacyclin and endothelium-derived hyperpolarising factor will be discussed briefly, since they are also potential pathophysiological candidates for Raynaud's disease, as will be addressed later.

## A.1. Endothelium-Derived Vasoconstrictors

### A.1.1. Endothelin

#### A.1.1.1 The discovery of endothelin

The first definitive evidence of an endothelium-derived vasoconstrictor factor came from Hickey and co-workers who, in 1985, demonstrated that the supernatant of cultured bovine endothelial cells possessed sustained vasoconstrictor activity, a finding which was confirmed by others in subsequent studies (Gillespie *et al.*, 1986; O'Brien *et al.*, 1987). In 1988, this factor was isolated and sequenced from cultured porcine endothelial cells by Yanagisawa *et al.*, and was found to be a 21 amino acid

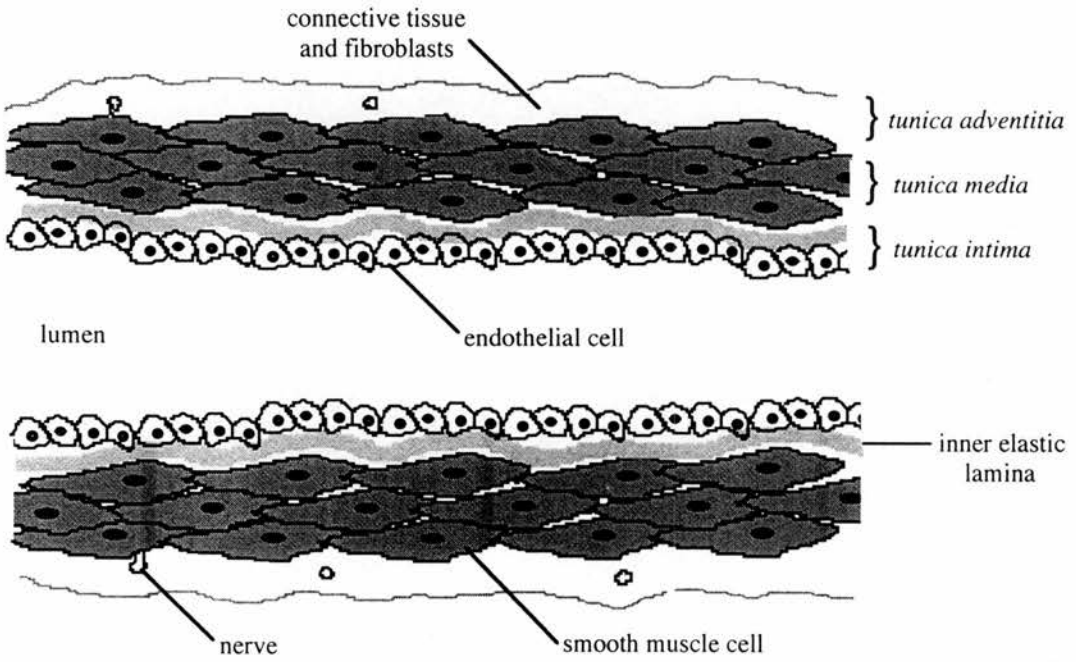



***Figure 1.1.*** *Schematic longitudinal section of a small artery showing the three layers of the vascular wall.*

peptide, which they named endothelin (Yanagisawa *et al.*, 1988). Endothelin (ET) is the most potent vasoconstrictor agent identified to date, and has a characteristically prolonged effect: Yanagisawa and colleagues (1988) showed that intravenous administration of ET caused a long-lasting pressor response in chemically denervated rats, the increase in arterial pressure being sustained for more than 60 minutes, in contrast to other vasoconstrictor hormones.

ET exists as a family of isopeptides; ET-1, the isoform originally identified by Yanagisawa *et al.* (1988), and ET-2 and ET-3, which are structurally and pharmacologically distinct (Inoue *et al.*, 1989) (Figure 1.2). The sarafotoxins (SRTX's), four peptides isolated from the venom of the Israeli burrowing asp, *Atractaspis engaddensis*, have a remarkable structural similarity with the ET's (Figure 1.2), and indeed share their potent effects (Kloog *et al.*, 1988). As can be seen from Figure 1.2, the structures of all three human ET isoforms and the four SRTX's (S6a, b, c and d; only S6c isoform shown) are identical at 10 of the 21 amino acids; ET-2 is the most similar to ET-1, differing by only 2 amino acids, whilst ET-3 differs by 6. All ET and SRTX isoforms have two intrachain disulphide bonds which link cysteine residues at positions 1, 15, 3 and 11. It is now recognised that these disulphide bridges are important in predicting receptor binding affinities (Nakajima *et al.*, 1989).

### A.1.1.2. Generation and metabolism of endothelin

ET-1 is the major form produced by vascular endothelial cells in humans, and is generated in response to hypoxia, vascular shear stress, and a range of other vasoactive mediators, including adrenaline (Yanagisawa *et al.*, 1988). The primary translation product of the human ET-1 gene is a 212 amino acid prepropeptide, containing a 17 amino acid signal sequence which is cleaved once the peptide is secreted from the nucleus (Inoue *et al.*, 1989a; 1989b). The synthesis of mature ET-1 from prepro ET-1 requires two enzymatic steps: firstly, dibasic amino acid endopeptidase(s) cleaves the prepropeptide at Lys52-Arg53 and Arg90-Arg91 residues

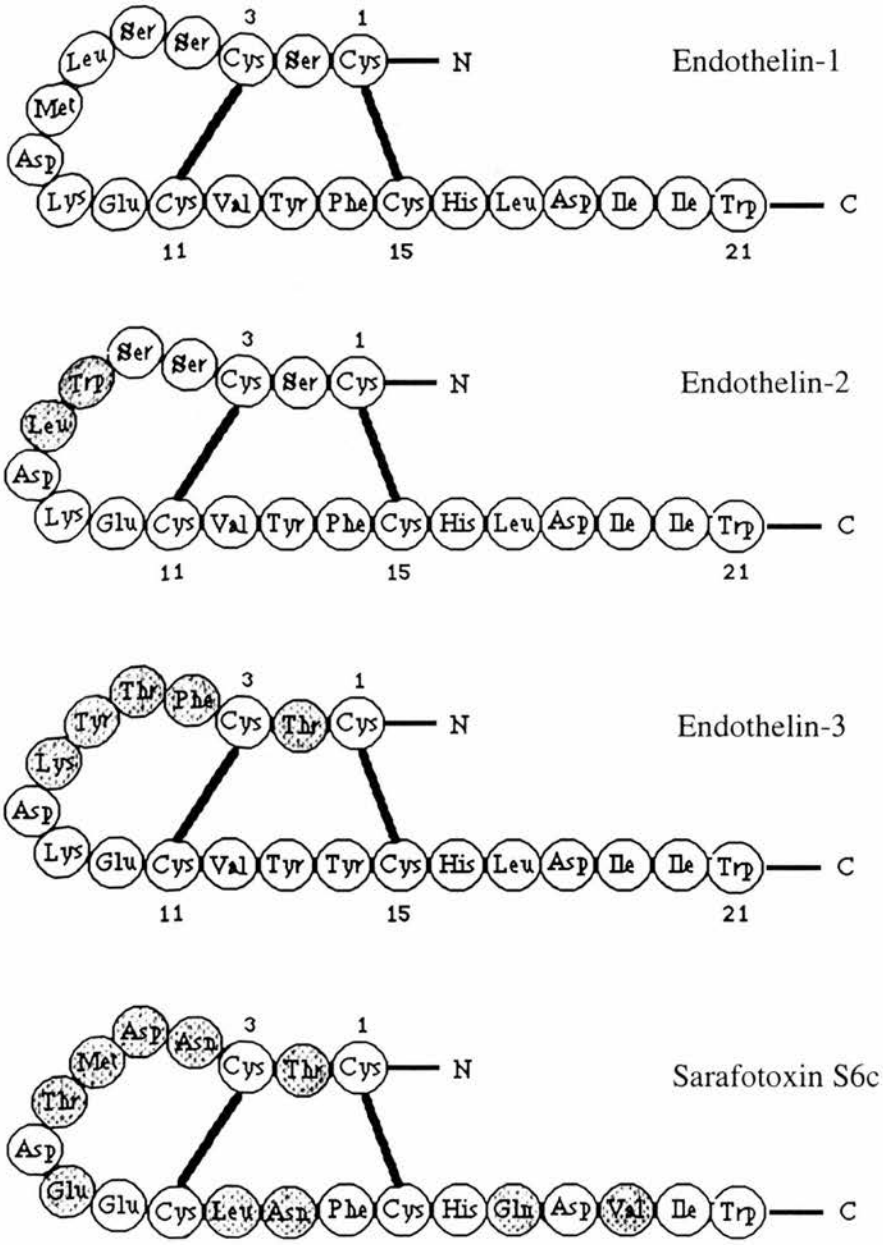



***Figure 1.2.*** *Amino acid sequences of the three human endothelin isoforms, and one of the sarafotoxin family (four highly homologous peptides isolated from snake venom). Amino acids differing from endothelin-1 are shaded, and the lines represent disulphide bridges linking cysteine residues.*

to form the 38 amino acid pro ET-1, or 'big ET-1', then cleavage at Trp21-Val22 by endothelin-converting enzyme (ECE), yields the 21 amino acid ET-1 (Figure 1.3).

Several candidates have been proposed for the activity of ECE; based on their sensitivity to metal ion chelators, pepstatin A and pH optima, the aspartyl and/or metalloprotease family were thought to be responsible for ECE activity (Ikegawa *et al.*, 1990; Lees *et al.*, 1990; Sawamura *et al.*, 1990). However, the aspartyl proteases were subsequently shown to be unlikely candidates, owing to the inability of aspartyl protease inhibitors to attenuate ET-1 secretion from cultured endothelial cells (Ikegawa *et al.*, 1990; Shields *et al.*, 1991). Neutral endopeptidase 24.11 may play a role in the conversion of big ET to the mature peptide, but it appears that it has a greater catalytic efficiency in degrading ET-1 (Abassi *et al.*, 1993) (see below). The most likely enzyme displaying ECE activity is the neutral, membrane-bound metalloprotease which is senstive to the inhibitor phosphoramidon (Matsumura *et al.*, 1990a; Okada *et al.*, 1990). Phosphoramidon has been shown to inhibit the pressor response to exogenous big ET-1 (Fukuroda *et al.*, 1990; Matsumura *et al.*, 1990b; McMahon *et al.*, 1991), and also big ET-2 and big ET-3 (Gardiner *et al.*, 1992b; Pollock *et al.*, 1993), inferring that the enzyme(s) which converts the other isoforms of big ET is likely to be similar to that involved in ET-1 formation. ECE is now thought to exist as a family of isoenzymes, with different selectivities for the big ET isoforms. ECE-1 and ECE-2 from animal and human sources have recently been cloned (Xu *et al.*, 1994; Emoto & Yanagisawa, 1995), and both are found to convert big ET-1 more efficiently than big ET-2 and big ET-3. Unlike ECE-1, which has optimum activity at the neutral pH of 6.8, ECE-2 has an acidic pH optimum of 5.5, and has a 200-fold greater affinity to phosphoramidon compared to ECE-1 (Emoto & Yanagisawa, 1995).

The metabolism of ET appears to occur by three different routes: enzymatic degradation, receptor-mediated clearance, and urinary excretion. Membrane bound neutral endopeptidase 24.11 (NEP) cleaves ET-1 into biologically inactive fragments

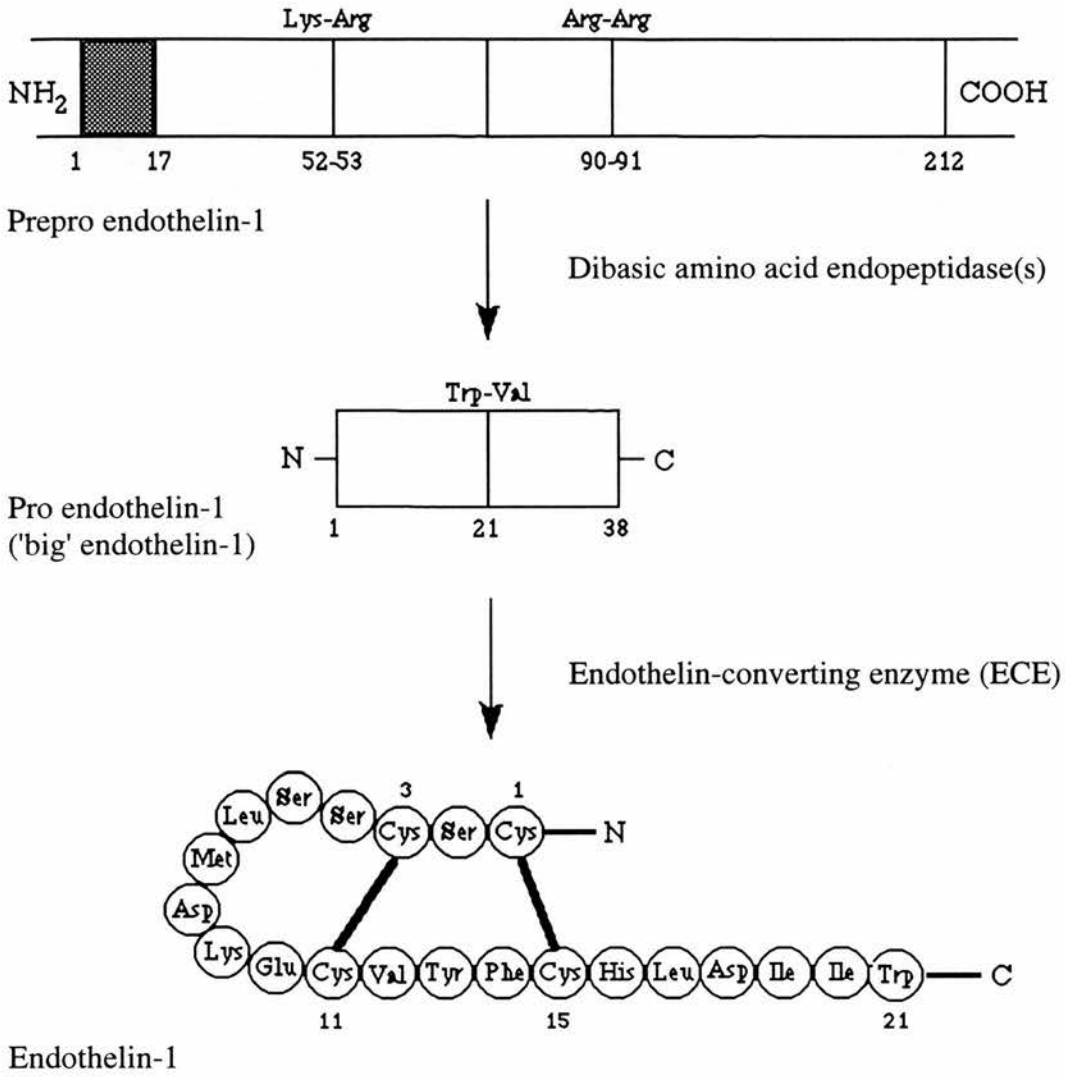



***Figure 1.3.*** *Proposed pathway for generation of endothelin-1. Cleavage of the signal peptide sequence (amino acids 1-17, shaded) occurs during the secretion of big endothelin-1 from the nucleus. Enzymatic conversion of big endothelin-1 to the mature peptide involves a dibasic amino acid peptidase(s) and endothelin converting enzyme (ECE).*

(Sokolovsky *et al.*, 1990; Vijaraghavan *et al.*, 1990; Fagny *et al.*, 1991). NEP is also responsible for the metabolism of other peptides, including bradykinin, atrial natriuretic peptide and substance P (Erdos & Skidgel, 1989). Although NEP-inhibition did not potentiate the response to exogenous ET-1 in rats (Pollock *et al.*, 1993), its physiological role in the metabolism of ET is supported by the results of human studies using inhibitors of NEP. Increased urinary excretion and plasma levels of ET-1 were found after inhibition of NEP by SQ-29,072 (Abbassi *et al.*, 1992), and brachial artery infusion of the NEP inhibitor, thiorphan, has been shown to cause vasoconstriction of the forearm vasculature (Haynes & Webb, 1994). When [$^{125}$I]-labelled ET is injected intravenously (Shiba *et al.*, 1989) or into the left ventricle (Anggård *et al.*, 1989) of anaesthetised rats, over 60% of ET-1 is removed during the first minute, the majority of which binds to the lung, followed by the kidney and liver. The authors suggest ET-1 binds to its receptors and is then internalised (Anggård *et al.*, 1989; Shiba *et al.*, 1989). It has been reported that intracellular proteases, such as deamidase, then degrade the internalised ET (Jackman *et al.*, 1992; 1993). Low affinity $ET_B$ receptors may be clearance receptors for high concentrations of ET (Clozel *et al.*, 1993). As mentioned, urinary excretion accounts for part of the clearance of ET; bilateral nephrectomy of rats has been shown to significantly reduce the disappearance of [$^{125}$I]-labelled ET-1 (Kohno *et al.*, 1989).

### A.1.1.3 Endothelin receptors and signal transduction mechanisms of endothelin

To date, two distinct ET receptor subtypes have been cloned from human cDNA: the $ET_A$ receptor, initially cloned by Arai *et al.* (1990) from bovine cDNA, is selective for ET-1, with binding affinity: ET-1>ET-2>>ET-3 (Adachi *et al.*, 1991; Hosoda *et al.*, 1991), and the $ET_B$ receptor, originally cloned from rat cDNA by Sakurai *et al.* (1990), which is non-selective, with binding affinity: ET-1=ET-2=ET-3 (Ogawa *et al.*, 1991). A third receptor subtype, $ET_C$, which is ET-3 selective (binding affinity: ET-3>ET-2=ET-1), has been cloned in the amphibian *Xenopus laevis* (Karne *et al.*, 1993), but has yet to be identified in the mammalian genome. The $ET_A$ and $ET_B$

receptor subtypes are structurally similar, having seven transmembrane domains characteristic of the G-protein-coupled receptor superfamily (Hosoda *et al.*, 1992; Arai *et al.*, 1993).

The short term actions of ET are thought to be mediated by several signal transduction mechanisms, involving G-protein-mediated activation of phospholipase C (PLC), which catalyses the formation of inositol trisphosphate ($IP_3$) and diacylglycerol (DAG) (Pang *et al.*, 1989; Griendling *et al.*, 1989). $IP_3$ induces the release of intracellular calcium ($Ca^{2+}$) from the sarcoplasmic reticulum, whilst DAG activates protein kinase C (PKC), which may sensitise contractile proteins to $Ca^{2+}$ via phosphorylation (Sunako *et al.*, 1989; Abe *et al.*, 1991). PKC may also stimulate sodium-hydrogen ion ($Na^+$-$H^+$) exchange by increasing the intracellular $H^+$ affinity for the $Na^+$-$H^+$-antiporter (Lonchampt *et al.*, 1991), which leads to alkalinisation and enhanced contractility of vascular smooth muscle cells, again, through sensitisation of contractile proteins to $Ca^{2+}$. Voltage-operated $Ca^{2+}$ channels are also involved in the sustained vasoconstrictor response to ET (Inoue *et al.*, 1990). In addition, there is evidence that ET-1 can stimulate arachidonic acid metabolism via activation of phospholipase $A_2$ ($PLA_2$) (De Nucci *et al.*, 1988; Reynolds *et al.*, 1989), resulting in the release of prostacyclin, prostaglandin $E_2$ and thromboxane $A_2$.

The wide use of selective agonists and antagonists *in vivo* confirm that $ET_A$ receptors, present on vascular smooth muscle cells, mediate vasoconstriction, whilst the $ET_B$ receptors on endothelial cells, cause vasodilatation. In addition, selective $ET_B$ receptor agonists, SRTX S6C and [Ala $^{1,3,11,15}$] ET-1, have been shown to increase blood pressure *in vivo*, which suggests that vasoconstrictor $ET_B$ receptors exist (Williams *et al.*, 1991; Douglas & Hiley, 1991; Clozel *et al.*, 1992; Gardiner *et al.*, 1992a). Further evidence for a constrictor $ET_B$ receptor comes from antagonist studies, where the $ET_A$ receptor antagonists, BQ-123 or FR 139317, are unable to fully block the pressor response to ET-1 (Ihara *et al.*, 1992; Bigaud & Pelton, 1992; McMurdo *et al.*, 1993;

Warner *et al.*, 1994; Gardiner *et al.*, 1994a). These results demonstrate that both $ET_A$ and $ET_B$ receptors mediate the pressor effects of ET. The physiological contribution of constrictor $ET_B$ receptors is presently uncertain, and it appears to vary depending on the size and type of vessel under study. In the pulmonary vasculature of the rat, $ET_B$ receptor agonists were able to induce a far greater contraction in resistance arteries compared to the larger pulmonary arteries (MacLean *et al.*, 1994), and their role appears to be more important in pulmonary resistance arteries compared to mesenteric resistance arteries in the rat (Deng *et al.*, 1995). Non-$ET_A$, non-$ET_B$ receptors may also be involved, although studies in conscious rats using the non-selective $ET_A/ET_B$ receptor antagonist, bosentan, find the major haemodynamic effects of ET-1, ET-2 and ET-3, and all those of big ET-1, are effectively antagonized (Gardiner *et al.*, 1994b), which is consistent with $ET_A$ and $ET_B$ receptors mediating the majority of the haemodynamic responses to the ETs.

ET receptors can be downregulated in response to increased levels of ET. Hirata and co-workers (1988) were first to demonstrate a reduction in [$^{125}$I]-ET-1 binding sites, and a reduced ability of ET-1 to induce a rise in intracellular $Ca^{2+}$, after pretreatment with ET-1 in cultured vascular smooth muscle cells.

#### A.1.1.4. Cardiovascular effects

ET has a wide range of actions throughout the body, including effects on the function of the kidney, lung, gut, central nervous system and neuroendocrine system. The focus of this section will be on the cardiovascular physiology of ET.

##### A.1.1.4.1. Pressor responses of endothelin

In addition to the prolonged pressor response to ET-1, originally demonstrated by Yanagisawa *et al.* in 1988, ET-2 and ET-3 also increase blood pressure in the rat (Inoue *et al.*, 1989a), although ET-3 evokes the least response and has the shortest duration of action. Similar pressor responses to the ETs have been demonstrated in

many species, including the guinea pig (Whittle *et al.*, 1989a; Braquet *et al.*, 1989), rabbit (Miyamori *et al.*, 1990), dog (Clarke *et al.*, 1989), pig (Pernow *et al.*, 1989), goat (Dieguez *et al.*, 1992), trout (Olson *et al.*, 1991) and man (Vierhapper *et al.*, 1990).

Studies using [$^{125}$I]-labelled ET-1 in the anaesthetised rat show that the sustained increase in arterial pressure occurs despite a rapid clearance of ET-1 from the circulation (Anggård *et al.*, 1989; Shiba *et al.*, 1989), inferring that the long lasting pressor response is due to an extremely slow dissociation of ET-1 from its receptors on vascular smooth muscle, as has been reported *in vitro* (Hirata *et al.*, 1988).

Big ET-1 causes a pressor effect of similar magnitude to that of ET-1 in the conscious rat (Gardiner *et al.*, 1991; McMahon *et al.*, 1991), and in anaesthetised rats (Matsumura *et al.*, 1990a), guinea pigs (Fukuroda *et al.*, 1990), and rabbits (D'Orleans-Juste *et al.*, 1991). In the anaesthetised pig, however, porcine big ET-1 shows a poor vasopressor activity compared to ET-1 (Hemsen *et al.*, 1991); this result may be due to the mode of administration of big ET-1, because the other studies cited used bolus injections, whilst Hemsen *et al.* gave infusions, or, it could also be due to species differences. The pressor activity of big ET-1 is dependent on its metabolism to mature ET-1 by ECE, because big ET-1 is 100 times less potent *in vitro* than ET-1 (Kashiwabara *et al.*, 1989). As described earlier, ECE has been identified as a phosphoramidon-sensitive metalloprotease (Matsumura *et al.*, 1990a); studies *in vivo* show phosphoramidon potently inhibits the pressor response of big ET-1 without affecting ET-1 induced hypertension (Fukuroda *et al.*, 1990; Matsumura *et al.*, 1990b; McMahon *et al.*, 1991). Vasoconstriction induced by big ET-1 in the mesenteric vascular bed is less sensitive to phosphoramidon than its vasoconstriction in renal and hindquarters vascular beds (Gardiner *et al.*, 1991). This suggests that conversion of big ET occurs locally rather than in the systemic circulation, with different vascular beds possessing varying degrees of enzyme activity. The precursors of ET-2 and ET-3

also result in vasoconstrictor actions similar to their mature forms when administered *in vivo* to conscious rats (Gardiner *et al.*, 1992b) and anaesthetised rats (Pollock *et al.*, 1993; Matsumura *et al.*, 1993), although the magnitude of the response is smaller than that of ET-2 and ET-3. Unlike studies in the rat, D'Orleans-Juste and colleagues (1991) found big ET-3 had no pressor response in the anaesthetised guinea pig. The contrasting findings *in vivo* may reflect species variation in the expressions of different ECE isoforms.

#### A.1.1.4.2. Depressor responses of endothelin

ET elicits a transient depressor response, lasting only a few minutes, which precedes the pressor effect and is most marked for ET-3 (Inoue *et al.*, 1989a). From studies using analogs of SRTX in rats, it appears to be the N-terminal amino group that determines the vasodilator activity of the peptide (Kitazumi *et al.*, 1990). When big ET-1 is given to conscious rats, the depressor response observed with the mature peptide is absent. The same is true when ET-1 is administered as an infusion rather than as a bolus dose (Gardiner *et al.*, 1989; Mortensen & Fink, 1990). The absence of the hypotensive effect is most likely to reflect the physiological response to endogenously generated ET, where plasma concentrations are much lower.

The transient depressor response to bolus administration of ET is thought to be mediated by endothelial generation of nitric oxide and prostacyclin from endothelial cells, after stimulation of the endothelial $ET_B$ receptor. Some studies using inhibitors of nitric oxide synthase (Whittle *et al.*, 1989b; Auberson *et al.*, 1991; Fozard & Part, 1992; Lerman *et al.*, 1992; Rogerson *et al.*, 1993; Filep *et al.*, 1993; Granstam *et al.*, 1993) and cyclo-oxygenase (De Nucci *et al.*, 1988; Filep *et al.*, 1991b; Rogerson *et al.*, 1993; Granstam *et al.*, 1993) have shown a significant reduction in the dilator effect, and sometimes a potentiation of the pressor effect of ET-1, indicating the constrictor action of ET is limited by the stimulated release of vasodilators (see below). Furthermore, the ability of $ET_A$ receptor antagonists to enhance the vasodilator activity

of ET again suggests a functional antagonism exists between constrictor and dilator actions. Others have found no major role for nitric oxide or prostacyclin in mediating the vasodilator response to ET *in vivo* (Gardiner *et al.*, 1989; 1990; Ohlstein *et al.*, 1990), suggesting that other endothelial mediators may be involved in some vascular beds. Plasma levels of the cardiac-derived vasodilator, atrial natriuretic peptide, are increased following intravenous injection of ET-1 in conscious rats (Stasch *et al.*, 1989), although this dilator is not thought to play a major role in the depressor action of ET-1 (Fozard & Part, 1990). It would seem unlikely that the endothelium-derived vasodilator, platelet-activating factor (PAF), mediates the initial hypotensive response to ET-1, since Filep *et al.* (1991a) have shown that pretreatment of conscious rats with selective PAF-receptor antagonists fails to affect the haemodynamic response to ET-1. Endothelium-dependent hyperpolarising factor (EDHF) has been proposed as another mediator of ET-induced dilatation, although few studies *in vivo* have been done. One such study demonstrated that glibenclamide, an inhibitor of ATP-dependent potassium channel activation, failed to block the depressor response to ET-1 (Lippton *et al.*, 1991), suggesting EDHF may not play a major role in this response, since EDHF has been reported to induce hyperpolarisation via the opening of ATP-dependent potassium channels (see review by Garland *et al.*, 1995). Glibenclamide does, however, inhibit the dilator response to ET-1 in the pulmonary circulation (Lippton *et al.*, 1991; Wong *et al.*, 1993), suggesting a role for EDHF in this vascular bed.

#### A.1.1.4.3. Endothelin and the maintenance of blood pressure

ET may play a role in maintaining normal resting blood pressure, since administration of phosphoramidon alone results in a significant fall in mean arterial pressure both in normotensive and in hypertensive rats (McMahon *et al.*, 1991). Similarly, intravenous infusion of the $ET_A$ receptor antagonist, BQ-123, decreases mean arterial pressure in normotensive (Pollock & Opgenorth, 1993) and hypertensive rats (Bazil *et al.*, 1992; Nishikibe *et al.*, 1993), and guinea pigs (Véniant *et al.*, 1994). ET has been shown to

contribute to basal vascular tone in the forearm of humans, after an increase in forearm blood flow was found following intra-arterial infusion of phosphoramidon or BQ-123 (Haynes & Webb, 1994). More recently, studies using systemic doses of the $ET_{A/B}$ receptor antagonist, TAK-044, reveal that mean arterial pressure is reduced in a dose-dependent manner after intravenous administration of this antagonist to healthy subjects (Haynes *et al.*, 1995). Thus, endogenous generation of ET-1 appears to contribute to the maintenance of blood pressure, implying a fundamental physiological role for this peptide in cardiovascular control.

#### A.1.1.4.4. Cardiac effects of endothelin

The coronary resistance bed is more sensitive to the effects of ET than other resistance beds, except for the kidney (Clozel & Clozel, 1989). ET-1 has been shown to be a potent and long-lasting constrictor of coronary arteries *in vivo* in animals, including the dog (Kurihara *et al.*, 1989a; Miller *et al.*, 1989; Wang *et al.*, 1991), rabbit (Hirata *et al.*, 1990), monkey (Clozel & Clozel, 1989), goat (Dieguez *et al.*, 1992) and pig (Pernow *et al.*, 1989; Ezra *et al.*, 1989), resulting in a marked fall in coronary blood flow and myocardial ischaemia. Coronary angiograms reveal these effects are primarily due to the contraction of small coronary arteries (Kurihara *et al.*, 1989a; Hirata *et al.*, 1990). ET has also been reported to lead to fatal ventricular arrhythmias (Ezra *et al.*, 1989), possibly via a distinct proarrhythmic effect separate from myocardial ischaemia.

Positive chronotropic and inotropic effects of ET have been found *in vitro*, but reports *in vivo* have been conflicting. Several studies find the intial hypotension produced by ET is associated with an increase in heart rate and cardiac output, whilst the pressor response causes bradycardia and a fall in cardiac output, which is probably due to a combination of systemic vasoconstriction, increasing afterload, and coronary vasoconstriction, causing myocardial ischaemia (Miller *et al.*, 1989; Yang *et al.*, 1991). In contrast, Garcia and collegues (1990) report positive inotropic actions of

ET-1, despite a rise in mean arterial pressure in conscious rats. The early inotropic effects most likely reflect a direct cardiac action of ET-1 (Gardiner *et al.*, 1990c). Ricou *et al.* (1992) found no effect of ET-1 on myocardial contractile function in pigs.

Chronotropic effects of ET-1 *in vivo* appear to be reflex in origin, since heart rate does not change after blockade of cardiac efferent neural mechanisms (Gardiner *et al.*, 1990c).

### A.1.2. Angiotensin II

Angiotensin II (ANG II) is a potent vasoconstrictor peptide generated by a two-step enzymatic reaction. Renin, which is secreted into the blood from the juxtaglomerular cells of the afferent arteriole in the kidney, catalyses the formation of the decapeptide, angiotensin I (ANG I), from the plasma substrate, angiotensinogen. The membrane-bound enzyme, angiotensin converting enzyme (ACE), found in high concentrations in the lung, then cleaves two amino acids from ANG I to form the octapeptide, ANG II. The known actions of ANG II, including vasoconstriction, result from the activation of $AT_1$ receptors (see review by Cockcroft *et al.*, 1995). The presence of renin, angiotensinogen, and ACE in the blood vessel wall suggests that, in addition to the circulating renin-angiotensin system, ANG II may be released locally from the vasculature and contribute to the maintenance of vascular tone (Caldwell *et al.*, 1976; Campbell & Habener, 1986; Samani *et al.*, 1987). Direct evidence of vascular production of ANG II comes from studies of isolated vessels in rats (Nakamaru *et al.*, 1986) and humans (Mizuno *et al.*, 1991), which reveal ANG II is generated despite the absence of circulating blood. It is unclear to what extent the vascular renin-angiotensin system contributes to the maintenance of vascular tone, because administration of locally acting doses of ACE inhibitors (Webb *et al.*, 1988) and renin inhibitors (Haynes *et al.*, 1993) have little effect on local blood flow or vascular tone. An important action of ANG II generated within the vasculature is the potentiation of

sympathetically mediated vasoconstriction (Zimmerman *et al.*, 1981; Cockcroft *et al.*, 1995).

### A.1.3. Vasoconstrictor cyclooxygenase products

Cyclooxygenase inhibitors, such as indomethacin, have been shown to prevent endothelium-dependent contractions induced by several mediators (Katusic *et al.*, 1988), suggesting metabolites of arachidonic acid are capable of eliciting contraction, either directly or indirectly. Some of these vasoconstrictor metabolites are discussed below.

#### A.1.3.1. Thromboxane $A_2$/Prostaglandin $H_2$

Thromboxane $A_2$ ($TXA_2$), which is generated predominantly in platelets, can also be synthesised in endothelial cells, through the action of two enzymes: cyclooxygenase, which catalyses the formation of the endoperoxides, prostaglandin $G_2$ ($PGG_2$) and prostaglandin $H_2$ ($PGH_2$), from arachidonic acid, and thromboxane synthetase, which converts $PGH_2$ into $TXA_2$ (see review by Schror, 1993). However, in blood vessels prostacyclin synthetase is more commonly found than thromboxane synthetase, thus favouring prostacyclin formation over that of $TXA_2$ (see below). Once bound to its $TXA_2/PGH_2$ (TP) receptor (see review by Pierce *et al.*, 1995), the vasoconstrictor and platelet aggregatory effects of $TXA_2$ are mediated through activation of PLC to elevate intracellular $Ca^{2+}$ through increased phosphoinositide turnover, membrane DAG and PKC (Seiss *et al.*, 1985; Brass *et al.*, 1987).

Several substances, including acetylcholine, 5-hydroxytryptamine (5-HT), and adenosine diphosphate (ADP), cause endothelium-dependent contractions which are inhibited by TP receptor antagonists or cyclooxygenase inhibitors (Auch-Schwelk *et al.*, 1990; Kato *et al.*, 1990), indicating the involvement of endoperoxides/thromboxanes in the contractile response. It remains unclear whether the endoperoxides/thromboxanes are released directly from the endothelium, or

alternatively, if an endothelium-derived signal is responsible for initiating their production in vascular smooth muscle cells.

#### A.1.3.2. Superoxide anions

Superoxide anions, or oxygen-derived free radicals ($O_2^-$), have been shown to be released from cultured endothelial cells (Rosen & Fremann, 1984). $PGH_2$ synthetase may be a source for these anions, since the conversion of $PGG_2$ to $PGH_2$ has been shown to generate oxygen-derived free radicals (Kukreja *et al.*, 1986). Endothelium-dependent contraction of the canine basilar artery is inhibited by superoxide dismutase, an $O_2^-$ scavenger, supporting a role for superoxide anions in mediating contraction of this vessel (Katusic & Vanhoutte, 1989). It would appear that superoxide anions cause contraction of vascular smooth muscle indirectly, either by stimulating the release of $PGH_2$ which then acts on TP receptors (Auch-Schwelk *et al.*, 1989; 1990), or through inactivation of the endothelium-derived dilator, nitric oxide (NO), since superoxide anions are avid scavengers of NO (Rubanyi *et al.*, 1986; Gryglewski *et al.*, 1986).

## A.2. Endothelium-Derived Vasodilators

### A.2.1. Endothelium-derived relaxing factor

#### A.2.1.1 The discovery of endothelium-derived relaxing factor

In 1980, Furchgott & Zawadzki demonstrated *in vitro* that acetylcholine (ACh) caused relaxation in arteries with an intact endothelium, but was unable to elicit relaxation in those which were denuded of their endothelium, and in fact caused contraction in these vessels due to a direct action on vascular smooth muscle (Furchgott & Zawadzki, 1980). These findings led them to believe that there existed either an electrical or humoral signal that could pass from the endothelium to the underlying smooth muscle, to cause dilatation in response to ACh. In order to determine the nature of this signal, Furchgott and colleagues (1984) set up a sandwich preparation of rabbit aorta, which comprised strips of endothelium-denuded vessels in very close contact with strips of intact vessels. Upon addition of ACh, both strips relaxed, showing that the factor

responsible for this relaxation could be transferred from one strip to another, i.e. was a diffusable humoral signal. This signal was named endothelium-derived relaxing factor (EDRF).

EDRF has a very short half-life, in the order of a few seconds, which made it very difficult to identify. Endothelium-dependent relaxation was shown to be inhibited by haemoglobin (Martin *et al.*, 1985). Gryglewski and co-workers (1986) demonstrated superoxide anions ($O_2^-$) inactivate EDRF, whilst the $O_2^-$ scavenger, superoxide dismutase, stabilises EDRF. The nitrovasodilators, such as glyceryl trinitrate (GTN) and sodium nitroprusside (SNP), activate soluble guanylate cyclase in vascular smooth muscle cells via the production of nitric oxide (NO) (Feelisch & Noack, 1987). EDRF was also shown to stimulate soluble guanylate cyclase (Forstermann *et al.*, 1986; Ignarro *et al.*, 1986). These observations led to the belief that EDRF could itself be NO. The identity of EDRF as NO would explain its short half-life (rapidly decays to nitrate and nitrite), its destruction by $O_2^-$ and enhancement by superoxide dismutase, the actions of the nitrovasodilators, and its inhibition by haemoglobin, since NO fits into the haem ring in exactly the same place as oxygen ($O_2$). Further evidence for NO being the EDRF came from superfusion cascade experiments by Palmer *et al.* (1987) who added bradykinin, a known releaser of EDRF, to strips of rabbit aortae. Their findings showed that the amount of NO released, measured by chemiluminescence, quantitatively accounted for the relaxation of the aortic strips produced by bradykinin-induced release of EDRF. Similar findings were reported by others in the same year (Hutchison *et al.*, 1987; Ignarro *et al.*, 1987; Khan & Furchgott, 1987). In addition, the rate of loss of relaxing activity with increasing time was similar for NO and EDRF (Palmer *et al.*, 1987). Similarities between the inhibitory effect of EDRF and NO on platelet aggregation were also demonstrated (Radomski et al., 1987). The above observations provide good evidence that EDRF is NO. Several other candidates, such as S-nitroso-L-cysteine (Myers *et al.*, 1990), have been proposed to be the EDRF, but the results of a recent study comparing the properties of several of these other

candidates with those of NO, further support the identity of EDRF as NO (Feelisch *et al.*, 1994) (Figure 1.4).

### A.2.1.2. Generation and metabolism of endothelium-derived relaxing factor

NO is synthesised within the vascular endothelium by nitric oxide synthase (NOS) acting on the amino acid substrate, L-arginine (Palmer *et al.*, 1988a; 1988b). Three distinct NOS enzymes have been identified to date: two constitutive, $Ca^{2+}$-calmodulin-dependent types present in the endothelium (and platelets) and neuronal tissue (Mayer *et al.*, 1989; Bredt *et al.*, 1991), and an inducible $Ca^{2+}$-independent type found in immune cells, vascular smooth muscle cells, endothelial cells and myocytes, which is expressed during immunological and inflammatory reactions (Kilbourn & Belloni, 1990). The three isoforms of NOS (endothelial, neuronal, and cytokine-inducible) are derived from three separate genes (for review see Sessa, 1994). NADPH, FAD and FMN are required as cofactors for each isoform, and tetrahydrobiopterin potentiates their activity (Mayer *et al.*, 1991; Yui *et al.*, 1991; Schmidt *et al.*, 1992). Endothelial NOS is activated by a rise in the endothelial intracellular calcium concentration (Mulsch *et al.*, 1989; Mayer *et al.*, 1989), which explains the endothelium-dependent relaxing effect of the $Ca^{2+}$-ionophore, A23187 (Ignarro *et al.*, 1987), and the various mediators of relaxation, including bradykinin and ADP, which release $Ca^{2+}$ from the sarcoplasmic reticulum via stimulation of the $IP_3$ pathway (Lambert *et al.*, 1986; Pirotton *et al.*, 1987). NO has been shown to inhibit its own synthesis through a negative feedback mechanism on NOS (Assreuy *et al.*, 1993). Griscavage and colleagues (1994) suggest that NO interacts with NOS-bound haem iron in the ferric state to inhibit the enzyme.

NO degrades within seconds to form nitrite ($NO_2^-$) and nitrate ($NO_3^-$):

$$2\ NO_2 + H_2O \text{ ------> } NO_2^- + NO_3^- + 2\ H^+$$

Wennmalm and co-workers (1993) report that one of the major metabolic pathways for NO is uptake into the red blood cells, where it is converted into nitrate and methaemoglobin. Nitrate then enters the plasma and is excreted via the kidney.

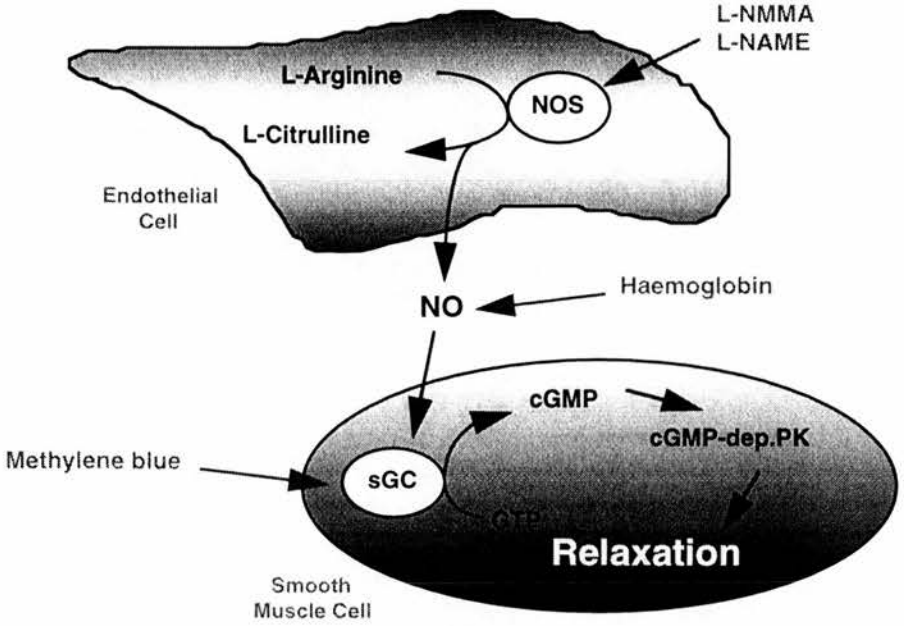
NO can also act as a free radical scavenger, neutralising superoxide anions to form nitrate:

$$NO + O_2^- \text{------>} NO_3^-$$

However, the reaction between NO and superoxide anions can result in the formation of highly toxic peroxynitrite anions ($ONOO^-$), which are cytotoxic and can cause considerable tissue damage (Beckman *et al.*, 1990).

### A.2.1.3. Nitric oxide receptors and signal transduction mechanisms of nitric oxide

Once NO has been formed it diffuses to the underlying smooth muscle, where it binds to the haem moiety associated with soluble guanylate cyclase and causes a conformational change, which activates the enzyme, leading to production of cGMP (Ignarro et al., 1986) (Figure 1.4). The cGMP in turn activates cGMP-dependent protein kinases (Fiscus *et al.*, 1984; Popescu *et al.*, 1985) which can then cause vasodilatation by several methods: for example, by inhibiting sarcolemmal calcium channels (Collins *et al.*, 1986; Ratz *et al.*, 1987) or activating sarcolemmal calcium extrusion pumps (Popescu *et al.*, 1985), to result in a net reduction of $Ca^{2+}$ influx; or by affecting the phosphorylation/dephosphorylation of myosin light chain contractile protein (Rapoport *et al.*, 1983). Recently, Bolotina and co-workers (1994) demonstrated that NO can cause smooth muscle relaxation independently of cGMP, through the direct activation of $Ca^{2+}$-dependent potassium channels. NO has also been shown to stimulate ADP-ribosylation of G-proteins, which enhances the activation of adenylate cyclase whilst inhibiting the activation of PLC, thus resulting in vasodilatation (Brune *et al.*, 1994).



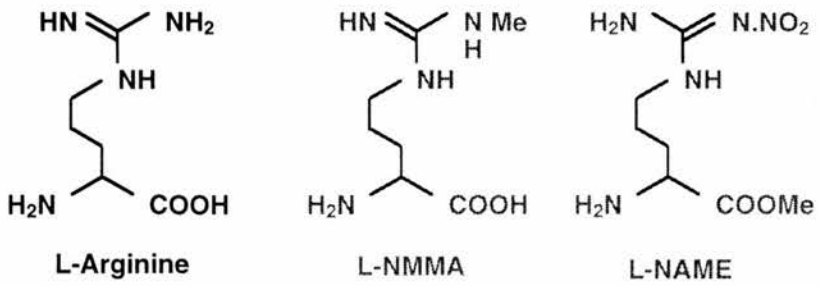




***Figure 1.4.*** *Schematic pathway for the generation of nitric oxide (NO). NO is produced from the enzymatic conversion of L-arginine to L-citrulline by NO synthase (NOS). After diffusing to the underlying smooth muscle, NO activates soluble guanylate cyclase (sGC), leading to the accumulation of cyclic guanosine monophosphate (cGMP). cGMP-dependent protein kinases then mediate smooth muscle relaxation. Inhibitors of the pathway are shown in grey: $N^G$-monomethyl L-arginine (L-NMMA) and $N^G$-nitro-L-arginine methyl ester (L-NAME) inhibit NOS; haemoglobin inactivates NO; and methylene blue prevents the activation of sGC.*

### A.2.1.4. Vasodilator effects of nitric oxide

NO is released in response to hypoxia, pulsatile flow and shear stress (Pohl *et al.*, 1986; Rubanyi *et al.*, 1986), and is the final common pathway for the action of many vasodilator substances. Increases in blood flow have been shown to potentiate the endothelium-dependent dilator response to ACh in the dog hindlimb (Miller & Vanhoutte, 1988) and the rabbit ear (Griffith *et al.*, 1987). Griffith & Edwards (1990) have reported that the greatest release of NO is in the large arterioles, which experience the highest levels of shear stress. NO is also released by endogenous vasoconstrictors, including ET (Warner *et al.*, 1989), and serves to modulate their actions (see below). Analogues of L-arginine, such as L-NMMA, act as antagonists of NOS, and have been found to cause vasoconstriction in resistance vessels (Vallance *et al.*, 1989), indicating that nitric oxide exerts a tonic dilator action opposing constrictor influences in the arterial circulation. Most veins produce little, if any, NO under resting conditions and are very sensitive to exogenous NO in the form of nitrovasodilators (Vallance *et al.*, 1989). This supersensitivity to NO could result from an increased amount of soluble guanylate cyclase in vascular smooth muscle cells, or an enhanced sensitivity of the enzyme, in the venous circulation.

### A.2.2. Prostacyclin

Prostacyclin, or prostaglandin $I_2$ ($PGI_2$), was discovered in 1976 by Moncada and colleagues (Moncada *et al.*, 1976). It is mainly generated in endothelial cells, although some can be formed in the smooth muscle, from arachidonic acid, through the action of cyclooxygenase and prostacyclin synthetase. Once bound to its IP receptor (see review by Pierce *et al.*, 1995), the vasodilatory and anti-aggregatory effects of prostacyclin are mediated through a rise in intracellular cyclic AMP concentrations in smooth muscle cells and platelets (Tateson *et al.*, 1977). Because many investigators have used cyclooxygenase inhibitors as part of a standard protocol, the contribution of $PGI_2$ endothelium-dependent dilatation has not been well documented. Pulsatile flow and vasoactive mediators such as bradykinin and thrombin stimulate $PGI_2$ synthesis (Piper & Vane, 1977). It appears that arteries can synthesise three to ten times more

$PGI_2$ than veins (Skidgel & Printz, 1978), and larger vessels produce more $PGI_2$ than the microvasculature (MacIntyre *et al.*, 1978).

### A.2.3. Endothelium-derived hyperpolarising factor

Bolton and colleagues (1984) first described an endothelium-dependent, carbachol-induced hyperpolarisation in the guinea pig mesenteric artery. The finding that NO was unable to change the membrane potential of vascular smooth muscle cells (Komori *et al.*, 1988), indicated that the endothelium probably releases a hyperpolarising factor which is distinct from NO (reviewed by Garland *et al.*, 1995). Direct evidence for the existence of a separate endothelium derived hyperpolarising factor (EDHF) comes from studies showing that inhibitors of EDRF, such as haemoglobin and methylene blue, inhibit vasorelaxation induced by acetylcholine, but do not alter endothelium-dependent smooth muscle hyperpolarisation (Chen *et al.*, 1988; Komori *et al.*, 1988; Chen & Suzuki, 1989). The fact that exogenous nitro-vasodilators produce relaxation with little or no change in smooth muscle membrane potential further supports this theory (Komori *et al.*, 1988). Studies using selective agonists and antagonists for the different subtypes of muscarinic receptor, have revealed that endothelial M1 receptors mediate transient relaxation by EDHF release, whilst endothelial M2 (now M3) receptors are responsible for relaxation via EDRF (Komori & Suzuki, 1987). As artery size decreases, it appears that EDHF accounts for a greater proportion of the relaxation than NO (Nagao & Vanhoutte, 1993).

## A.3. Interactions between endothelium-derived relaxing and contracting factors

### A.3.1.Endothelin and nitric oxide

Experiments have shown that removal of the endothelium, or addition of inhibitors of NO, potentiates the contractile response to ET-1 and ET-3, indicating that ET stimulates the release of EDRF/NO (Warner *et al.*, 1989; Whittle *et al.*, 1989b; Auberson *et al.*, 1991; Fozard & Part, 1992, Lerman *et al.*, 1992). This effect is most

marked for ET-3, and is mediated through endothelial cell $ET_B$ receptors (Warner *et al.*, 1989; 1992; Hirata *et al.*, 1993). ET production is inhibited by NO via a cGMP-dependent mechanism (Boulanger & Luscher, 1990; Warner *et al.*, 1992). When the NO-synthase inhibitor, $N^G$-nitro-L-arginine methyl ester (L-NAME), is administered to anaesthetised rats, it produces a rise in mean arterial pressure (Richard *et al.*, 1995). This is not solely due to removal of basal dilator tone, because prior administration of the ET-receptor antagonists, BQ-123 or bosentan, resulted in a 50% reduction of the L-NAME-induced pressor response, implying that basal NO-mediated inhibition of ET release occurs (Richard *et al.*, 1995).

### A.3.2. Endothelin and prostacyclin

Indomethacin, but not L-NMMA, was able to prevent the ET-1-induced endothelium-dependent relaxtion in the isolated perfused rat mesentery, suggesting prostacyclin or $PGE_2$ is released in response to ET-1 (Dohi & Luscher, 1991). Similarly, ET-1 stimulates the release of dilator prostaglandins *in vivo*, as demonstrated by the potentiation of the pressor reponse to ET-1 in the presence of indomethacin (De Nucci *et al.*, 1988; Filep *et al.*, 1991b; Rogerson *et al.*, 1993; Granstam *et al.*, 1993). ET-3 has been shown to stimulate the synthesis and release of prostacyclin in cultured endothelial cells (Emori *et al.*, 1991b). Yokokawa and co-workers (1991) have found ET-3 -induced formation of ET-1 in cultured human endothelial cells is augmented by indomethacin. Similarly to NO, it would therefore appear that prostacyclin can inhibit ET release and/or synthesis.

### A.3.3. Nitric oxide and prostacyclin

Relaxations produced by exogenous prostacyclin are potentiated in the presence of the endothelium (Shimokawa *et al.*, 1988). Furthermore, this potentiation is inhibited by haemoglobin, implying prostacyclin induces the release of NO. It is, of course, possible that synergistic mechanisms are responsible for the enhanced relaxation. Prostacyclin production, however, has been shown to be inhibited by NO in cultured bovine endothelial cells (Doni *et al.*, 1988).

### A.3.4. Endothelin and angiotensin II

The contractile response to ANG II is augmented through an endothelium-dependent mechanism, which is most likely to be the release of ET, because phosphoramidon, BQ-123 or an ET antibody, prevents this augmentation (Dohi *et al.*, 1992; Webb *et al.*, 1992). The fact that ANG II and ET increase blood pressure in a synergistic manner, suggests a common pathway exists in the pressor mechanism of the two peptides (Yoshida *et al.*, 1991; 1992); common signalling mechanisms appear to exist for the delayed mitogenic action of ANG II and ET (Weber *et al.*, 1994). *In vitro*, ANG II has been shown to stimulate ET production in cultured endothelial cells (Dohi *et al.*, 1992; Chua *et al.*, 1993), smooth muscle cells (Scott-Burden *et al.*, 1991), cardiomyocytes (Ito *et al.*, 1993), and mesangial cells (Kohno *et al.*, 1992). Emori and co-workers (1991a) propose ANG II-induced stimulation of ET release is mediated by mobilisation of intracellular $Ca^{2+}$ and activation of PKC. The results of subsequent studies provide further evidence for this mechanism (Kohno *et al.*, 1992). The positive interaction between ANG II and ET occurs both ways; in cultured endothelial cells, ET enhances the conversion of ANG I to ANG II (Kawaguchi *et al.*, 1990). Interestingly, ANG II has been shown to downregulate ET-1 receptors in vascular smooth muscle cells (Roubert *et al.*, 1989) and *in vivo* in the pithed rat (Guillon *et al.*, 1991), revealing a cross-desenstisation exists between ANG II and ET-1.

## B. The Vascular Endothelium In Pathophysiology

Endothelial dysfunction has been implicated in a number of vascular conditions. However, it is difficult to predict the pathophysiological role of individual endothelium-derived factors, given that intrinsic regulatory mechanisms exist between dilator and constrictor substances released from the endothelium (see above), and the fact that pressor agents, adrenaline and ANG II (Yanagisawa *et al.*, 1988) for example, can enhance the release of endothelium-derived vasoconstrictors, such as ET. Discerning what is the cause, and what is an effect of the disease, is therefore difficult; increases in levels of vasoconstrictors may be secondary to a dysfunction in

release/action of vasodilators. Many studies have measured plasma concentrations of vasoconstrictors as a means of identifying potential pathophysiological candidates. Plasma levels of ET are not very informative, since experiments in cultured endothelial cells found ~80% of ET was preferentially secreted abluminally (Wagner *et al.*, 1992), implying ET is unlikely to be acting as a circulating hormone. Indeed, normal plasma levels of ET, in the low picomolar range in healthy humans (Davenport *et al.*, 1990), are lower than concentrations required to elicit contraction in isolated vascular rings (Yanagisawa *et al.*, 1988).

Despite the difficulties highlighted above, there is strong evidence that certain disease states are related to an imbalance between endothelium-derived dilator and constrictor function. Studies using ECE-inhibitors and ET-receptor antagonists, and inhibitors of the prostacyclin and NO pathways, have supported a role for the endothelium in numerous pathological conditions. Some of these conditions are listed in Table 1.1. As this thesis addresses the vasospastic disorder of Raynaud's disease, the role of the endothelium in the related conditions of migraine and variant (Prinzmetal's) angina is discussed in detail.

## B.1. Vasospastic disorders

### B.1.1. Migraine

The debate as to whether migraine headaches are primarily the result of a vascular or neurological disturbance remains unresolved, but there clearly is a decrease in regional blood flow preceding migraine (Skinhoj & Paulson, 1969; Skyhoj-Olsen *et al.*, 1987). The cause of cerebral vessel vasospasm is unknown. The role of ET in mediating cerebral vasoconstriction has been investigated by Farkkila and colleagues (1992), and their interesting, though limited, results show that plasma ET-1 levels are raised in migraine patients during an attack, compared to controls. There is to date, no

***Table 1.1.*** *Pathophysiological conditions associated with endothelial dysfunction*

| | |
|---|---|
| Myocardial infarction | Miyauchi *et al.*, 1989; Omland *et al.*, 1994 |
| Chronic heart failure | McMurray *et al.*, 1992; Wei *et al.*, 1994 |
| Ischaemic stroke | Ziv *et al.*, 1992 |
| Atherosclerosis | Zeiher *et al.*, 1991 |
| Hypercholesterolaemia | Bath & Martin, 1991; Chowienczyk *et al.*, 1992 |
| Subarachnoid haemorrhage | Masaoka *et al.*, 1989; Suzuki *et al.*, 1992 |
| Migraine | Farkkila *et al.*, 1992; Olesen *et al.*, 1994 |
| Variant angina | Quyyumi *et al.*, 1992; Matsuyama *et al.*, 1991 |
| Raynaud's phenomenon | Zamora *et al.*, 1990; |
| Hypertension | Panza *et al.*, 1990; Kohno *et al.*, 1990 |
| Pulmonary hypertension | Stewart *et al.*, 1991 |
| Pre-eclampsia | Dekker *et al.*, 1991; Florijn *et al.*, 1991 |
| Chronic renal failure | Koyama *et al.*, 1989 |
| Acute renal failure | Tomita *et al.*, 1989 |
| Diabetes mellitus | Takahashi *et al.*, 1990; Johnstone *et al.*, 1993 |
| Septicaemic shock | Petros *et al.*, 1991 |
| Gastric ulcer | Wallace *et al.*, 1989 |
| Bronchial asthma | Mattoli *et al.*, 1991 |

information regarding basal ET levels between migraine attacks. ET is also implicated in subarachnoid haemorrhage-induced vasospasm (Matsumura *et al.*, 1991; Suzuki *et al.*, 1992; Clozel *et al.*, 1993) favouring the possibility that ET may play a pathogenetic role in migraine.

NO may also be involved in the pathophysiology of migraine. Subjects given continuous intravenous infusion of the endothelium-independent vasodilator, glyceryl trinitrate (GTN), had reproducible, dose-dependent headaches during infusion, which rapidly disappeared after infusion stopped (Iversen *et al.*, 1989). GTN infusions caused more severe headaches in migraine sufferers compared to controls (Olesen *et al.*, 1993), and similar findings were reported in studies using histamine, an endothelium-dependent dilator (Krabbe & Olesen, 1980). It appears therefore, the 'fault' in migraine sufferers lies downstream from NO itself, because the sensitivity to NO is increased in migraineurs in response to both histamine and GTN, and is thus independent of NO-synthase. A possible candidate for the 'fault' is soluble guanylate cyclase, which may display increased activity, or another enzyme, cofactor or receptor in the NO-mediated pathway (Olesen *et al.*, 1994).

### B.1.2. Variant angina

Variant angina, first described by Prinzmetal and co-workers in 1959, results from transient vasospasm of the coronary arteries (Prinzmetal *et al.*, 1959; Dhurandhar *et al.*, 1972). Endothelial dysfunction has been reported in patients with variant angina who have a reduced vasodilator reserve (Quyyumi *et al.*, 1992). Impaired NO release probably accounts for this dysfunction because vasodilatory responses of the coronary microvasculature to the endothelium-dependent dilator, acetylcholine, are depressed in these patients, whilst sodium nitroprusside, an endothelium-independent dilator, produces similar responses to those found in patients without a reduced vasodilator reserve, indicating the smooth muscle sensitivity to NO is normal (Quyyumi *et al.*, 1992).

ET has been implicated in the pathophysiology of variant angina, but there is some controversy as to its exact role. Basal plasma ET-1 concentrations are elevated between vasospastic attacks in patients with variant angina (Toyo-oka *et al.*, 1991), but it is unclear if ET is primarily involved in intiating coronary spasm, because Toyo-oka and colleagues (1991) found there was a transient decrease in plasma ET levels from coronary sinus blood during an attack, in contrast to Matsuyama *et al.* (1991), who reported increased levels 20 to 30 seconds after arteriographic evidence of spasm. Although ET itself may not be directly responsible for initiating the spasm, it may be important in influencing the sensitivity of coronary arteries to other constricting agents; subthreshold concentrations of ET-1 have been shown to potentiate the contraction of isolated human coronary arteries to noradrenaline and 5-HT (Yang *et al.*, 1990; Chester *et al.*, 1992).

### B.1.3. Raynaud's disease

Raynaud's disease is characterised by intense vasospasm of the extremities, particularly the digits, in response to cold exposure or marked emotion. Evidence suggests a temperature-dependent disorder of the vascular endothelium, whereby either overproduction of a vasoconstrictor factor, or underproduction of a dilator factor, might be a critical factor underlying the pathogenesis of Raynaud's (see under Pathophysiology: D.1.10. for a detailed discussion).

### B.1.4. Generalised vasospasm

An association exists between the vasospastic conditions of migraine, variant angina, and Raynaud's disease (Miller *et al.*, 1981; Zahavi *et al.*, 1984; Kaiser, 1992; O'Keeffe *et al.*, 1992; 1993), in that there is a significantly higher incidence of Raynaud's in patients with migraine and/or variant angina, suggesting that there may be a vascular defect common to these conditions. Interestingly, patients with variant angina show an enhanced forearm vasoconstriction to the cold pressor test, further supporting the hypothesis of a generalised vasospastic disorder in these patients

(Nakamura *et al.*, 1984). Incidently, in common with Raynaud's disease, migraine has a higher incidence in women (Linet & Stewart, 1987), again supporting a generalised disorder. As discussed for the individual conditions, the endothelium is a favoured candidate for the pathogenesis of this generalised vasospasm.

An increased prevalence of Raynaud's disease and migraine has also been reported in subjects with ocular vasospasm (Phelps & Corbett, 1985; Guthauser *et al.*, 1988; Drance *et al.*, 1988), a condition which is thought to be an important factor in the pathophysiology of low-tension glaucoma (Gasser, 1989). Drance and co-workers (1988) demonstrated in finger blood flow experiments that patients with vasospastic diseases of the eye had an abnormal response to cold. The results of a recent study suggest that ET-1 may be associated with ocular vasospasm (Sugiyama *et al.*, 1995).

## C. The Digital Circulation

Before describing Raynaud's disease and its pathophysiology in detail, it is worthwhile to provide an overview of the anatomy and physiology of the digital circulation. The symptoms of Raynaud's disease occur predominantly in the fingers, but the following information is also relevant to the other extremities which can be affected, including the toes, ears, and nose.

### C.1. The arterial circulation

The blood supply to the hands and fingers comes from the ulnar and radial arteries. From Figure 1.5, it can be seen that each digit receives two digital arteries. The blood supply to the thumb arises directly from the radial artery, and not the palmar arch as for the fingers, which accounts for the fact that the thumb is often spared from vasospastic attacks in Raynaud's patients. Not all the fingers may be involved in an attack, which suggests that the vasospasm occurs in vessels distal to the common palmar digital arteries, since they supply branches to two fingers (Figure 1.5).

## C.2. Arteriovenous anastomoses

The digits contain a large number of arteriovenous (A-V) anastomoses, or shunts, in addition to the capillary circulation (Hale & Burch, 1960; Sherman, 1963). The coiled blood vessels which form A-V anastomoses have thick muscular walls but very little elastic tissue, and tend to be either fully open or closed. Their function is to regulate body temperature. When body temperature rises, A-V anastomoses open to allow large amounts of blood to flow through subcutaneous veins, from which heat can dissipate. During exposure to cold, blood is directed through the peri-arterial venae comites, bypassing superficial veins in the skin, as a result of the A-V anastomoses closing (Figure 1.6). The A-V anastomoses are under the control of both central and circulating stimuli. Closure of A-V anastomoses is brought about by sympathetic adrenergic stimulation, in a reflex response to cold or to mental stress, acting via $\alpha_2$-adrenoceptors, and by circulating 5-HT acting on 5-$HT_2$ receptors, noradrenaline and angiotensin II (Coffman & Cohen, 1988). Dilatation of A-V anastomoses results from decreased sympathetic stimulation, histamine acting on $H_1$ and $H_2$ receptors, and $\beta$-adrenoceptor stimulation (Coffman & Cohen, 1988). Interestingly, Raynaud's disease affects only skin areas that possess A-V anastomoses.

## C.3. Cold vasoconstriction

The primary mechanism controlling digital blood flow is the sympathetic nervous system; stimulation of sympathetic nerves results in vasoconstriction via $\alpha$-adrenoceptors, and withdrawal of their activity induces vasodilatation. The degree of sympathetic nerve activity is controlled centrally through the medulla oblongata, which contains the pressor and depressor vasomotor centres, which are themselves influenced by the hypothalamus and other higher centres of the cortex. Serotonergic vasoconstrictor mechanisms also play a role. 5-HT and $\alpha$-adrenoceptor function is covered in a subsequent section and so will not be discussed in detail here (see under Pathophysiology D.1.3. and D.1.4.).

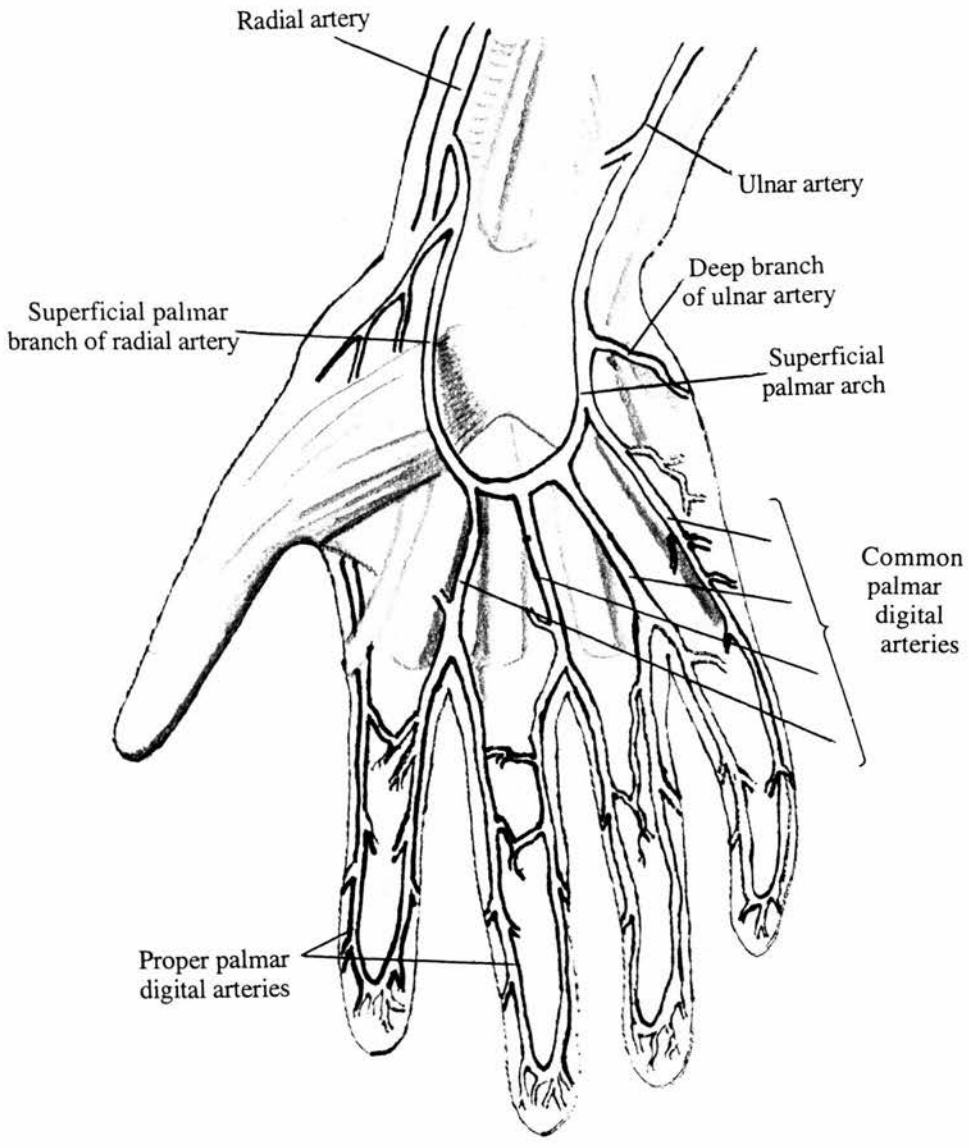



***Figure 1.5.*** *The arterial circulation of the hand, showing the superficial palmar arch and its branches.*

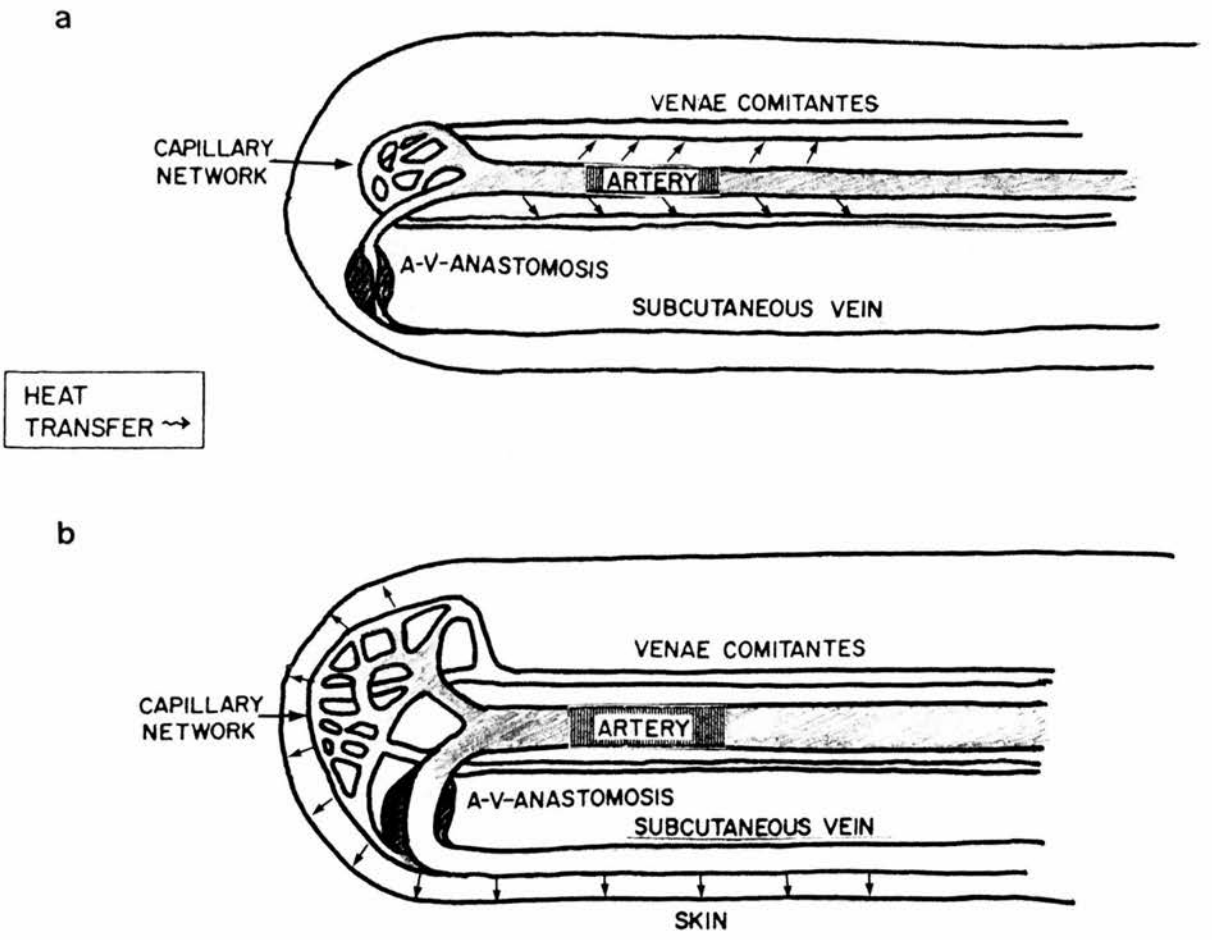



***Figure 1.6.*** *Diagram of the arteriovenous (A-V) anastomoses of the digits. When the body is cold, heat is conserved (a), and heat is lost when the body is hot (b).*

### C.4. Cold vasodilatation

Local cooling produces vasoconstriction of the fingertip, but at temperatures of less than 10°C, vasoconstriction alternates with periods of vasodilatation (Grant & Bland, 1931), most likely as a means of preserving tactile sensation in the digits. This phenonemon is largely due to local axon reflex mechanisms, whereby cold-induced stimulation of sensory C-fibre neurons causes the release of various neurotransmitters, including calcitonin gene-related peptide (see below), which then act on the vascular smooth muscle of blood vessels to cause vasorelaxation. Other vasodilatory mechanisms in response to cold may include the stimulation of β-adrenoceptors (Cohen & Coffman, 1981), histamine receptor activation (Coffman *et al.*, 1984) and a cholinergic vasodilator mechanism in the capillary vascular bed of the digits (Coffman & Cohen, 1987).

## D. Primary Raynaud's Disease

Raynaud's phenomenon takes its name from the French physician, Maurice Raynaud, who first described its symptoms in his thesis entitled *'De l'asphyxie locale et de la gangrene symetrique des extremities'* ('On local asphyxia and symmetrical gangrene of the extremities'), which he submitted in 1862. Raynaud's phenomenon is now recognised as a common condition, occurring in up to 20% of the population, and more often in females, affecting eight times as many women as men (Reviewed by Cotton & Khan, 1986; Belch, 1991; Coffman, 1991; Lally, 1992). The reported prevalence of the phenomenon varies according to a survey's diagnostic criteria, and the climatic conditions of where the survey is carried out; warmer climates tend to favour a lower percentage of the population being affected. The primary condition, where no underlying disorder is found, is known as Raynaud's disease, but it can occur secondary to several other conditions, in which case it is referred to as Raynaud's phenomenon or syndrome (Allen & Brown, 1932). Raynaud's disease most commonly affects the fingers and toes, but can affect any cold-exposed extremity, including the nose, ears and tongue (Cooke, 1991). Characteristically,

during an attack there is a triphasic response, although some patients do not experience all of the three phases. Initially, on exposure of the digits to cold, or in response to emotional stimuli, there is vasoconstriction producing extreme *pallor*, followed by the development of *cyanosis* through reduction in tissue oxygenation, and then *rubor* on return of the circulation. This last phase is often accompanied by intense local discomfort or pain. Vasoconstriction in response to cold exposure is a physiological response, which is exaggerated in subjects with Raynaud's disease. Currently, neither the mechanism of cold-induced vasoconstriction, nor of Raynaud's, is fully understood and, although many treatments are available for patients with Raynaud's and may be useful in individual subjects, none are effective in the majority of patients (Cooke & Nicolaides, 1990) (see later).

### D.1. Pathophysiology

At present, the pathophysiology of primary Raynaud's disease remains unresolved, despite its first description over 130 years ago (Raynaud, 1862). Outlined below are some of the possible candidates that may be responsible for the condition.

#### D.1.1. Sympathetic nervous system

Raynaud's disease was originally attributed to overactivity of the sympathetic nervous system (Raynaud, 1862). Evidence to support this theory comes from work by Peacock (1959), who reported elevated catecholamine levels in wrist venous blood taken from patients with Raynaud's disease. Excess sympathetic activity is also implied from the fact that postural changes produce an exaggerated vasoconstrictor response in patients with Raynaud's (Olsen *et al.*, 1987), and sympathectomy, surgical cutting of the sympathetic nerves, can often prevent attacks, although usually it is only the toes which benefit from this treatment (Janoff *et al.*, 1985).

There are, however, a number of studies which dispute the role of the sympathetic nervous system in the pathogenesis of Raynaud's disease. Among them is work

carried out by Sir Thomas Lewis (1929) who demonstrated the nerves could not be solely responsible for vasospastic attacks (see below). In contrast to the findings of Peacock (1959), Kontos and Wasserman (1969) did not find an increase in plasma or urinary catecholamine levels in primary Raynaud's patients. Perhaps the strongest evidence against sympathetic vasoconstrictor overactivity comes from a study in Raynaud's patients, where microelectrodes were used to record sympathetic nerve activity in one hand, whilst the other was cooled by ice water immersion (Fagius & Blumberg, 1985). The authors were unable to find an abnormal increase in sympathetic outflow. Although sympathetic activity *per se* may not be increased in Raynaud's, receptors on the vascular smooth muscle may be abnormally sensitive to the neurotransmitters released from the sympathetic nerve terminals (see section D.1.3.).

Despite the conflicting results regarding the role of the sympathetic nervous system in Raynaud's, the fact remains that emotional stress can induce vasospastic attacks in some people, indicating that there is some neuronal basis for the initiation of an attack.

### D.1.2. Local fault

In 1929, Lewis' studies indicated that the theory of sympathetic overactivity, first proposed by Raynaud in the 1800's, could not be the full explanation for the cause of the disease. He effectively denervated the hand using a local anaesthetic at the ulnar nerve, and was still able to produce vasospastic attacks in Raynaud's patients (Lewis, 1929), a finding which has since been reproduced by Freedman *et al.* (1989). Lewis concluded that the 'fault' in patients with Raynaud's lay in the digital arteries themselves, which exhibit abnormal sensitivity to cold. Potential candidates for this 'local fault' are introduced in some of the sections below.

### D.1.3. Alpha-adrenoceptors

α-Adrenoceptors were originally subdivided on an anatomical basis: $\alpha_1$-adrenoceptors were found postjunctionally, with $\alpha_2$-adrenoceptors located prejunctionally. The postjunctional vascular $\alpha_1$-adrenoceptor was thought to be solely responsible for vasoconstriction. However, it is now evident that, in addition to inhibiting further release of noradrenaline from nerve terminals by a negative feedback mechanism, the $\alpha_2$-adrenoceptor is also present postjunctionally, where it can mediate vasoconstriction (Stevens & Moulds, 1981; McGrath, 1982; Flavahan *et al.*, 1984). Among the many different vessels studied, the human digital artery has been shown to possess vasoconstrictor $\alpha_1$- and $\alpha_2$-adrenoceptors postjunctionally (Stevens & Moulds, 1986). Flavahan and colleagues (1987) have demonstrated that a greater proportion of vasoconstriction is mediated by $\alpha_2$-adrenoceptors as blood vessels become more distal, a finding that has been confirmed by others (Hughes *et al.*, 1987; Nielsen *et al.*, 1989).

Increased $\alpha_2$-adrenergic function has been implicated in the pathophysiology of Raynaud's disease. In 1978, Janssens & Vanhoutte reported that cooling potentiated contractions to noradrenaline in cutaneous veins from dogs, and suggested an increased affinity of postjunctional α-adrenoceptors may have been responsible for this effect (Janssens & Vanhoutte, 1978). Subsequent *in vitro* studies, using selective agonists and antagonists of $\alpha_1$- and $\alpha_2$-adrenoceptors in canine (Flavahan *et al.*, 1985; Flavahan & Vanhoutte, 1986) and human veins (Bodelsson *et al.*, 1990; Harker *et al.*, 1990), indicate that it is the $\alpha_2$-subtype which is augmented by cooling. Similar findings have been reported from *in vitro* arterial studies (Ekenvall *et al.*, 1988; Harker *et al.*, 1991). Interestingly, the results from one group suggest that the sensitivity of *both* receptor subtypes is increased at 24°C in human skin arteries (Gomez *et al.*, 1991), but this potentiation was only seen when using low concentrations of phenylephrine, an $\alpha_1$-adrenoceptor agonist, and clonidine, an $\alpha_2$-adrenoceptor agonist. At higher doses, the contractile responses were reduced during cooling,

which led Gomez and co-workers to conclude that cooling depresses the maximum contractility of human skin arteries to α-adrenergic stimulation. The same group have carried out studies in rabbit ear and femoral arteries (Garcia-Villalon *et al.*, 1992), and find that cooling inhibits the contraction to α-adrenoceptor agonists (through increased availability of NO in the ear arteries, and depressed sensitivity of α-adrenoceptors in the femoral artery). However, it has previously been demonstrated that $\alpha_2$-adrenoceptors make little contribution to the contraction of rabbit ear arteries (Harker & Vanhoutte, 1988) or deep, non-cutaneous vessels like the femoral artery (Vanhoutte & Flavahan, 1986), which would explain the lack of a potentiating effect of cooling on the responses to α-adrenergic agonists in these preparations.

Several clinical studies have investigated the effect of temperature on α-adrenergic vasoconstriction, and the role of α-adrenoceptors in Raynaud's disease. Reflex sympathetic vasoconstriction to body cooling has been demonstrated to be primarily mediated by $\alpha_2$-adrenoceptors in healthy subjects (Coffman & Cohen, 1988). Freedman and colleagues (1992) infused phenylephrine and clonidine into the brachial artery of healthy male volunteers, and found cooling abolished the vasoconstriction to phenylephrine, but potentiated that induced by clonidine, this effect being inhibited by the $\alpha_2$-adrenoceptor antagonist, yohimbine. In similar studies, brachial artery infusions of phenylephrine and clonidine produced a greater decrease of finger blood flow in Raynaud's patients compared to controls, suggesting that the sensitivity and/or number of α-adrenoceptors is increased in patients with Raynaud's disease (Freedman *et al.*, 1989; Coffman & Cohen, 1990). This is supported by studies showing platelets from Raynaud's patients have an increased number of $\alpha_2$-adrenoceptors (Keenan & Porter, 1983; Edward *et al.*, 1987). In a recent study, Freedman and co-workers (1995) assessed the effectiveness of prazosin and yohimbine in attenuating cold-induced vasospastic attacks in Raynaud's patients, and found that $\alpha_1$-adrenoceptor activation is not required for inducing vasospasm, which further supports the findings outlined above.

The sensitivity and/or density of peripheral vascular $\alpha_1$- and $\alpha_2$-adrenoceptors have been shown to be lower in women compared to men (Freedman *et al.*, 1987), a finding which does not correlate with the higher incidence of Raynaud's disease in women, if the $\alpha_2$-adrenoceptor is to be considered as a primary pathophysiological candidate. Although there is strong evidence to support a pathogenetic role for $\alpha_2$-adrenoceptors in Raynaud's disease, it is possible that another factor(s) may be simultaneously involved, with vasospastic attacks resulting from the combination of $\alpha_2$-adrenoceptors and other factor(s). An example of such a factor is an abnormality of endothelium-dependent dilator function (see below), which would lead to a reduced ability of the endothelium to oppose vasoconstriction mediated by $\alpha$-adrenoceptors.

### D.1.4. 5-Hydroxytryptamine

5-hydroxytryptamine (5-HT, serotonin) receptors are present in the finger vascular bed (Coffman & Cohen, 1988; Blauw *et al.*, 1991). The 5-$HT_2$ subtype is responsible for mediating vasoconstriction and platelet aggregation. Cooling has been found to enhance the contraction induced by 5-HT in canine cutaneous veins (Vanhoutte & Shepherd, 1970), and patients with Raynaud's disease have an increased finger vasoconstrictor response to 5-HT compared to controls (Coffman & Cohen, 1990). Biondi and colleagues (1988) found increased levels of plasma 5-HT in Raynaud's patients compared to controls, the highest being in those with the secondary form, but other workers have failed to detect such an increase (Coffman & Cohen, 1994). Ketanserin, a selective 5-$HT_2$ antagonist with weak $\alpha_1$-adrenergic antagonistic actions, relieves cold-induced vasoconstriction in Raynaud's patients, but does not alter the frequency of occurrence of the vasoconstriction (Seibold, 1985). This suggests that although 5-HT may be involved in the maintenance of digital artery vasospasm, perhaps released on platelet activation, other factors are probably responsible for the initiation of an attack.

### D.1.5. Platelets

Platelets are essential for haemostasis and are involved in the repair of damaged blood vessels, through a process involving their adhesion, activation and aggregation, which results in the formation of a haemostatic plug. When platelets are activated they release a number of vasoactive and trophic factors, including 5-HT, thromboxane $A_2$ ($TXA_2$) and platelet activating factor (PAF). An increased level of $TXB_2$, the stable metabolite of $TXA_2$, and an increased sensitivity of platelets to ADP and collagen, has been reported in patients with Raynaud's disease, although both effects are more marked in patients with secondary Raynaud's phenomenon (Wilkinson *et al.*, 1989). In contrast, others have reported no increase in the activation of platelets in Raynaud's disease (Seibold & Harris, 1985). It is difficult to determine whether raised $TXA_2$ and increased activity of platelets are causes, or merely effects of Raynaud's, because platelets both release, and are in turn regulated, by eicosanoids. Aggregated platelets have been shown to contract human digital artery strips (Moulds *et al.*, 1984), and platelet-induced contraction of canine arteries is potentiated by cooling (Lindbland et al., 1984), indicating a potential involvement of platelets in cold-induced vasospasm. However, it would appear from the results of Seibold & Harris (1985) and Wilkinson *et al.* (1989) that platelets are unlikely to have a pathogenetic role in Raynaud's disease. The fact that ticlopidine, a drug which inhibits platelet aggregation, had no effect on the frequency or duration of vasospastic attacks in Raynaud's patients provides further evidence against a primary involvement of platelets in this condition (Destors et al., 1986). Platelets probably have a greater role in secondary Raynaud's phenomenon, where endothelial damage promotes their adhesion and subsequent aggregation.

### D.1.6. Calcitonin gene-related peptide

Peptidergic nerves are known to innervate the skin microvasculature and appear to be involved in cold vasodilatation. Vasodilatation occurs through a local axon reflex mechanism, whereby stimulation of cold receptors on the terminals of cutaneous

sensory neurons triggers the release of neurotransmitter peptides, which then act on the adjacent cutaneous microvessels. Calcitonin gene-related peptide (CGRP) is a potent vasodilator released from such nerves (O'Halloran & Bloom, 1991). By acting on endothelial receptors linked to adenylate cyclase, CGRP increases cAMP levels, which in turn stimulate the synthesis of NO, inducing relaxation in the vascular smooth muscle through cGMP production (Marshall, 1992).

The digital skin of patients with Raynaud's disease has been shown to have a deficiency in CGRP-containing neurons (Bunker *et al.*, 1990; Terenghi *et al*, 1991), but the ability of these nerves to mediate an axon reflex response to histamine is not diminished in Raynaud's patients (Brain *et al.*, 1990). Furthermore, the responsiveness of the vasculature to CGRP does not appear to be reduced in Raynaud's disease since Brain and co-workers (1990) found in forearm blood flow studies, comparable vasodilator respsonses to exogenous CGRP in patients with Raynaud's disease and in control subjects, whilst Shawket *et al.* (1989) demonstrated a hypersensitivity to CGRP in Raynaud's patients, which perhaps reflects depressed endogenous generation of CGRP These results suggest that the coupling mechanism between cold-stimulation and the axon reflex response may be dysfunctional in these patients. A fault in the CGRP cold-induced vasodilatory mechanism would reduce the counterbalancing effect on vasoconstriction, and could therefore result in the vasospasm found in Raynaud's disease.

### D.1.7. Blood viscosity

Blood viscosity is determined by haematocrit, plasma viscosity and red cell deformability. Several investigators have found increases in whole blood viscosity in patients with Raynaud's disease (Tietjen *et al.*, 1975; Goyle & Dormandy, 1976; Blunt *et al.*, 1980), but haematocrit and red cell deformability do not appear to be altered (Rustin *et al.*, 1985; Challenor *et al.*, 1987). Plasma viscosity is raised in Raynaud's patients, which correlates with elevated plasma levels of fibrinogen (Tietjen *et al.*,

1975). The contraceptive pill, which is associated with a higher incidence of Raynaud's phenomenon (see below), increases blood viscosity through increasing fibrinogen levels. Jarret (1976) found that fibrinogen levels decreased and symptoms improved, when patients stopped taking the pill.

It should be noted that an increase in whole blood viscosity at low temperatures is a physiological phenomenon, and patients with Raynaud's disease do not appear to have any hyperviscosity during cooling. It is therefore unlikely that blood viscosity is of pathogenetic importance in Raynaud's, but merely exacerbates the condition by further reducing the blood flow through constricted arteries during vasospasm.

### D.1.8. Sex hormones

Raynaud's disease is more prevalent in women, suggesting hormonal influences are involved. Anecdotal evidence supports this: often a remission of symptoms is found during pregnancy; vasospastic attacks are exacerbated at different times during the menstrual cycle in some patients; the menopause is sometimes linked with the development of Raynaud's or, alternatively, the disappearance of symptoms in women whose Raynaud's disease began at puberty; and females taking the contraceptive pill have been reported to have a higher incidence of Raynaud's disease.

Oestrogen is known to enhance the sensitivity of small arteries to noradrenaline and adrenaline (Altura, 1975), by affecting the synthesis, uptake and release of catecholamines in the neurovascular junction (Vanhoutte *et al.*, 1981). Progesterone has the opposite effect, inhibiting smooth muscle contraction by promoting the uptake of calcium into the sarcoplasmic reticulum of smooth muscle cells (Carsten, 1979). Receptor density is also changed by the sex hormones: in rabbit uterus oestrogen increases the number of $\alpha$-adrenoceptors, favouring vasoconstriction, whilst progesterone decreases $\alpha$-adrenoceptors and increases the number of $\beta$-adrenoceptors, thus favouring vasodilatation (Roberts *et al.*, 1977). Recent evidence supports an

interaction between female sex hormones and the endothelium. In 1988, Williams *et al.* reported that treatment of female spontaneously hypertensive rats (SHR) with 17β-oestradiol significantly enhanced endothelium-dependent relaxation. Later studies in SHR found that pregnancy restored endothelium-dependent relaxation, compared to the diminished responses in nonpregnant animals (Ahokas *et al.*, 1991). Oestrogen has been shown to potentiate endothelium-dependent and -independent relaxation in the forearm vasculature of postmenopausal women (Gilligan *et al.*, 1994). Sex hormones appear to alter the expression of ET receptors: studies in human endometrial tissue have shown changes in the ratio of $ET_A$ and $ET_B$ receptors occur during the menstrual cycle (O'Reilly *et al.*, 1992).

In addition to their indirect effects on vascular tone, sex hormones may also have a direct effect: specific oestrogen- and progesterone-receptors have been identified on animal (Nakao *et al.*, 1981; Horwitz & Horwitz, 1982) and human vascular smooth muscle cells (Campisi *et al.*, 1987; Ingegno *et al.*, 1988). These receptors have been shown to mediate changes in the electrical properties, and contractility, of coronary vascular smooth muscle cells (Harder & Coulson, 1979).

Thermal entrainment studies, where one hand is alternately warmed and cooled whilst blood flow in the other hand is monitored, show the reactivity of peripheral blood vessels is influenced by the menstrual cycle (Lafferty *et al.*, 1985), with the maximum entrainment occuring when oestrogen levels are at their highest. During the peri-ovulatory period, normal woman displayed Raynaud's-like responses to thermal entrainment, in that the rapid post-sympathetic vasodilatation was delayed or absent (De Trafford *et al.*, 1984). However, Bartelink *et al.* (1994) examined the effects of single oral doses of oestrogen and progesterone on finger skin circulation, and found the female sex hormones had minor effects on finger blood flow, in both female controls and Raynaud's patients.

It would certainly appear that sex hormones do have a role in Raynaud's disease, but another factor must be responsible for causing the condition, since not all females suffer from vasospasm, and some men do, albeit in a smaller proportion. Lafferty *et al.* (1985) suggest that the women who do suffer from Raynaud's may have an exaggerated vascular response to the fluctuations in female sex hormones and, indeed, the fluctuations themselves may be abnormal. A plausible candidate(s) for this exaggerated vascular response may be one of the other factors outlined in this section.

### D.1.9. Hereditary or familial factor

There is no conclusive evidence that there is a hereditary factor in primary Raynaud's disease since few studies have addressed this issue. Greenwood (1976) reported a case study of three generations of a family, in which the inheritance of Raynaud's disease was compatible with an autosomal dominant transmission. It should be remembered that familial occurences can be due to the Raynaud's phenomenon being secondary to another disorder, such as connective tissue disease (see later) which can be hereditary, or indeed, the Raynaud's may be secondary to different etiologies in the same family.

### D.1.10. The vascular endothelium

Endothelial involvement in Raynaud's disease was first implicated after Zamora and colleagues (1990) performed cold provocation studies in patients with Raynaud's, and found an exaggerated increase in ET-1 concentrations in venous blood draining the cold-challenged arm when compared to the control arm, and with responses from healthy control subjects. Basal ET-1 levels were also elevated in Raynaud's patients, a finding which has been supported by others (Biondi *et al.*, 1991). In addition, a rapid increase in plasma ET-1 during the cold-pressor test in healthy subjects has been reported (Fyhrquist *et al.*, 1990). These results are consistent with a role for ET-1 in mediating the prolonged cold-induced vasospasm of Raynaud's disease. The rapid increase of ET-1, within minutes during cold exposure (Fyhrquist *et al.*, 1990),

implies ET is released from a pre-formed pool. Evidence to support the presence of such ET-secretory stores is limited at present, though their existence has been reported in rabbit (Milner *et al.*, 1990) and bovine (Harrison *et al.*, 1993) aortic endothelial cells. Measurements of big ET-1/C-terminal fragment concentrations in plasma would help to confirm the source of ET-1 produced during cooling; an increase in big ET-1/C-terminal fragment levels would imply *de novo* synthesis of ET-1, making ET release from secretory granules seem unlikely. Such studies have yet to be carried out.

Several groups did not find such an increase in ET-1 concentrations during cold exposure, thus forming some controversy as to whether ET is primarily involved in the pathogenesis of Raynaud's disease. Bottomley & Goodfield (1994) found no significant difference in basal or cold-challenged plasma ET-1 levels in Raynaud's patients when compared to controls. Smits and colleagues (1991), who also found basal ET-1 levels to be the same in control versus Raynaud's subjects, argue that Zamora *et al.*'s (1990) findings of increased venous ET-1 concentrations in blood draining the cold-challenged arm, can be explained by a decrease in forearm blood flow, resulting in decreased dilution, rather than an actual rise in ET-1 production. However, Zamora and co-workers (1990) accounted for this by demonstrating Raynaud's subjects consistently had a higher rise in ET-1 concentrations compared to controls, despite similar falls in pulsatility in both groups. Another study which disputes the involvement of ET in mediating cold-induced vasoconstriction, found no change in circulating ET-1 levels in healthy subjects, during a 2-hour stay in a room cooled to 10°C (Hassi *et al.*, 1991). The authors suggest that to see a rise in ET-1, the cold stimulus might have to be more intense than that experienced with whole-body cold exposure; cold-induced release of ET-1 is perhaps mediated through nociceptors, which are stimulated during cold-pressor tests (Klement & Arndt, 1992). An important finding of this study, was that circulating atrial natriuretic peptide (ANP) was increased during cold-exposure (Hassi et al., 1991). ET-1 is known to stimulate the release of ANP *in vitro* and *in vivo* (Stasch *et al.*, 1989; Ohman *et al.*, 1990). Cold-

induced release of ET-1 may be responsible for the rise in ANP, with the changes in ET-1 being too low to detect because, as explained in a previous section, measuring plasma levels of ET is not a good predictor of its pathogenetic role in disease, since ET is preferentially secreted abluminally (Wagner *et al.*, 1992), and is therefore unlikely to be acting as a circulating hormone, with raised levels merely reflecting over-spill from its release in the vasculature. Cimminiello *et al.* (1991) dismiss their findings of higher ET baseline levels in Raynaud's patients as a result of endothelial damage, stating the concentrations found are too low to elicit vasoconstriction. However, the concentration of ET at the endothelium-smooth muscle interface is probably a lot higher than plasma levels of ET (low picomolar range in healthy humans; Davenport *et al.*, 1990), which are indeed lower than concentrations required to elicit contraction of vascular smooth muscle *in vitro* and *in vivo* (Yanagisawa *et al.*, 1988). Furthermore, it should be remembered that subpressor doses of ET can potentiate vasoconstriction to other factors, such as noradrenaline and 5-HT (Yang *et al.*, 1990).

Further support for the involvement of ET in Raynaud's disease comes from experiments showing that ET-1 causes vasoconstriction of isolated perfused simian digital arteries (Haniuda *et al.*, 1991). Since the simian digital artery forms branches to the skin, the authors propose that its vascular reactivity is an important determinant of skin circulation. When ET-1 was injected intradermally into rabbit (Brain *et al.*, 1988) and human skin (Hughes *et al.*, 1989), it caused vasconstriction, as observed from the pronounced blanching at the site of injection. Autoradiographic experiments have demonstrated the presence of both $ET_A$ and $ET_B$ receptors in the microvessels of human skin (Knock *et al.*, 1993), and *in vivo* studies, using selective ET-receptor antagonists, have revealed that it is the $ET_A$ receptor which is primarily involved in the regulation of vascular tone in human skin microvasculature (Wenzel *et al.*, 1994). Interestingly, the ET-receptor density was found not to differ between patients with Raynaud's disease and controls, but was increased in patients with Raynaud's secondary to systemic sclerosis (Knock *et al.*, 1993). Given the reports of elevated

plasma levels of ET in Raynaud's disease, one could reasonably have predicted to find receptor down-regulation. Since the ET-receptors are not reduced in number, Knock and colleagues (1993) suggest that increased concentrations of plasma ET will result in enhanced contraction, and may therefore contribute to the vasospasm found in Raynaud's disease.

More direct evidence of a role for the endothelium in cold-induced vasoconstriction is provided by studies of cutaneous blood vessels, using the rabbit ear artery (Monge *et al.*, 1991). Here, the responses to ET-1 are temperature dependent, and under physiological conditions the vasoconstriction to ET-1 is attenuated by an increase in NO production during cooling. If blood vessels in patients with Raynaud's disease lack this NO-mediated inhibitory function of the endothelium during cooling, increased production and exaggerated vasoconstriction to ET-1 might lead to prolonged vasospasm. From the above observations, it appears that overproduction of ET-1, reduced production of NO, or a combination of these effects, might account for the vasospasm seen in Raynaud's disease.

As already mentioned, there is a higher incidence of migraine, variant angina and ocular vasospasm in patients with Raynaud's disease, suggesting that there may be a common vascular defect in these conditions (see above). Thus, the experimental evidence implicating the involvement of endothelial dysfunction in these related vasospastic conditions (e.g. Nakamura *et al.*, 1984; Toyo-oka *et al.*, 1991; Farkkila *et al.*, 1992), supports its role in the pathogenesis of Raynaud's disease.

## E. Secondary Causes of Raynaud's Phenomenon

When Raynaud's disease is known to be caused by an underlying disorder, it is referred to as Raynaud's phenomenon (Allen & Brown, 1932). There are numerous disorders associated with secondary Raynaud's phenomenon (Table 1.2), the most common of which are discussed below.

***Table 1.2.*** *Some secondary causes of Raynaud's phenomenon*

| |
|---|
| **Connective tissue diseases**<br>Systemic sclerosis, systemic lupus erythematosus, CREST syndrome, mixed connective tissue disease, rheumatoid arthritis, Sjögren's syndrome, dermatomyositis, polymyositis |
| **Drugs or chemicals**<br>β-adrenoceptor antagonists, ergotamines, bleomycin, cisplatin, vinblastine, oral contraceptives, vinyl chloride |
| **Occupational**<br>Vibration white finger or hand-arm vibration syndrome |
| **Occlusive arterial disorders**<br>Thrombosis, atherosclerosis, carpal tunnel syndrome, thoracic outlet syndrome |
| **Blood abnormalities**<br>Cryoglobulinaemia, cold agglutinins, polycythaemia |
| **Miscellaneous**<br>Hypothyroidism, tumours, diabetes mellitus, chronic renal failure |

### E.1. Connective tissue diseases

The most frequent conditions underlying the cause of Raynaud's phenomenon are those with immunological abnormalities, in particular the connective tissue diseases, the commonest of which is systemic sclerosis, or scleroderma. Ninety percent of patients with scleroderma have vasospastic attacks typical of Raynaud's phenomenon

(Tuffaneilli & Winkelmann, 1961), which are usually associated with CREST syndrome (subcutaneous **c**alcification, **R**aynaud's phenomenon, **e**sophageal dysfunction, **s**clerodermatous skin thickening, and **t**elangiectasia). Other connective tissue disorders asociated with Raynaud's phenomenon include systemic lupus erythematosus, rheumatoid arthritis and Sjögren's syndrome. Unlike primary Raynaud's disease, where there is no conclusive evidence that structural changes exist, the digital arteries in patients with connective tissue disorders are prone to intimal thickening and medial hypertrophy, sometimes resulting in occlusion of one or more digital arteries (Scott *et al.*, 1961; Laws *et al.*, 1963; Norton & Nardo, 1970).

### E.2. Drugs

The β-adrenoceptor antagonists, commonly used in hypertension, are the most common group of drugs known to induce Raynaud's phenomenon (Marshall *et al.*, 1976), possibly through an enhanced sensitivity of α-adenoceptors in peripheral vessels (White & Udwadia, 1975; Cohen & Coffman, 1980), or a reflex peripheral vasoconstriction induced by the decrease in cardiac output following β-adrenoceptor blockade. Drugs used in chemotherapy, such as bleomycin, cisplatin, and vinblastine, can induce secondary Raynaud's phenomenon, perhaps via induced hyperreactivity of the sympathetic nervous system (Hansen *et al.*, 1990). Ergotamine, which is effective against migraine headaches, causes vasospasm by acting on α-adenoceptors, although the attacks are likely to be more prolonged than those associated with Raynaud's disease, and can lead to digital gangrene (Cameron & French, 1960).

### E.3. Vibration white finger

Raynaud's phenomenon resulting from work involving the repeated use of vibrating instruments and tools, such as pneumatic drills, is termed vibration white finger (VWF) (or sometimes hand-arm vibration syndrome, HAVS). VWF was first described in Italian miners by Loriga (1911). Since then, a large proportion of workers using vibratory tools have been reported to develop vasospastic symptoms

characteristic of Raynaud's phenomenon (Cherniack, 1990). Lloyd Davies and colleagues believed that vibration only induces Raynaud's phenomenon when experienced in conjunction with cold exposure; body cooling and cold pressor tests did not produce symptoms of Raynaud's phenomenon in workers using vibratory tools in hot climates, such as Singapore (Lloyd Davies *et al.*, 1957). This theory is supported by Hellstrom and Anderson (1972), who report the highest incidence of VWF occurs in outdoor workers. Prolonged use of vibratory tools has been associated with peripheral neuropathy and neuromuscular dysfunction, particularly in the dominant hand (Marshall *et al.*, 1954). The pathogenesis of VWF is unknown, though reflex sympathetic hyperactivity and release of local hormones have been suggested (Olson *et al.*, 1975; Gemne, 1982).

## F. Treatment

### F.1. General measures

For the majority of patients with Raynaud's disease, where there is no obvious underlying disorder, preventative measures, such as avoiding the cold and other provocative stimuli, are more important than drug therapy. Although sufferers are advised to keep their whole body warm, electrically heated gloves and socks are found by many to be particularly helpful in preventing vasospastic attacks. Avoidance of tobacco smoking is also advised because it constricts digital blood vessels through sympathetic stimulation and reflexly via chemoreceptor stimulation. Smokers with Raynaud's phenomenon have been reported to have a greater smoking-induced decrease in digital blood flow, compared to non-smoking Raynaud's patients (Goodfield *et al.*, 1990). Interestingly, a recent study has found plasma ET-1 levels are increased within 10 minutes of cigarette smoking in healthy male subjects, together with a rise in systolic blood pressure (Haak *et al.*, 1994), whilst a study by Bodis & Haregewoin (1994) shows salivary NO production is reduced in smokers. Thus, smoking-induced vasoconstriction may be mediated through a change in ET and NO production, in addition to stimulation of the sympathetic nervous system.

## F.2. Drug therapy

Pharmacological intervention is usually reserved for Raynaud's patients who find their attacks are severe or are an intolerable inconvenience to their daily lives. The assessment of treatment in Raynaud's is made difficult due to the strong influence of factors such as the possibility of an underlying disorder, weather conditions, psychological stimuli, hormonal status in females, and smoking status. Although measures can be taken to standardise conditions within an individual study, this is difficult to ensure for trials carried out in different centres, and is a possible reason for the reported differences in effectiveness of therapies between individual research groups. A further confounding factor is the difficulty in provoking reproducible vasospastic attacks which correlate with those experienced by patients outside the laboratory. The effectiveness of the treatment on these vasospastic attacks then has to be objectively assessed by choosing a suitable means of measurement, which may not always be in agreement with the subjective report given by the patients themselves.

Some of the common pharmacological treatments presently used in Raynaud's disease are briefly reviewed below. Potential therapeutic approaches for the future are also addressed.

### F.2.1. Calcium channel antagonists

The calcium-channel antagonists are currently the first line of drug treatment for patients with Raynaud's disease, but are only effective in approximately 50% of patients. Nifedipine in the slow-release form is the most commonly used, owing to its preferential peripheral (over cardiac) vasodilator action (Smith & Rodeheffer, 1985; Finch *et al.*, 1988). Prophylactic sublingual nifedipine is an effective alternative in patients with predictable attacks or who are intolerant of long-term nifedipine treatment (Wollersheim *et al.*, 1987). Although the calcium-channel blockers' primary mode of action is to reduce the amount of calcium entering the smooth muscle cell, and thereby

decrease contractility, they have been reported to have additional effects: α-adrenoceptor antagonist activity, antiaggregatory action on platelets, and a beneficial effect on red blood cell deformability (Dale *et al.*, 1983; Timmermans *et al.*, 1983; Malamet *et al.*, 1985). Unfortunately, the side effects from calcium-channel blockers are considerable, including headaches and dizziness, flushing, palpitations and fluid retention, and may prevent their use in some patients.

### F.2.2. 5-$HT_2$ receptor antagonists

5-HT receptor antagonists prevent the vasoconstriction and platelet aggregation induced by 5-HT. Ketanserin has been the most widely investigated selective 5-$HT_2$ antagonist in Raynaud's disease, but naftidrofuryl, another 5-$HT_2$ blocker has also been examined (Haavik Nilsen, 1979; Gaylarde *et al.*, 1980). Ketanserin has been found to be beneficial in Raynaud's disease (Stranden *et al.*, 1982; Meloni *et al.*, 1987; Brouwer *et al.*, 1987; 1990), although at doses used clinically it may also be blocking $\alpha_1$-adrenoceptors and histamine $H_1$ receptors (Trenk *et al.*, 1983). However, Coffman and colleagues (1989) report in their large, international randomized trial, that the magnitude of the improvements by ketanserin in Raynaud's patients were small, and indeed, Longstaff *et al.* (1985) found the drug did not have any beneficial effects in their study. Seibold and co-workers have shown ketanserin reduces the severity but not the frequency of vasospastic attacks in patients with Raynaud's disease, suggesting 5-HT is involved in the maintenance of vasospasm, but other factors are important in the initiation of an attack (Seibold & Jageneau, 1984; Seibold & Terregino, 1986). The opposite was found in the trial by Coffman and colleagues (1989); the frequency of attacks was reduced, with no difference to the severity or duration. However, in thermometry studies carried out by Arosio *et al.* (1991), in addition to an increase in skin temperature, a decrease in the duration *and* the number of vasospastic attacks in patients was found, with no effect on their severity. Thus, the exact role of 5-HT in Raynaud's disease remains obscure. Side effects of ketanserin,

though less common than with nifedipine, include dizziness, sedation, dry mouth or eyes, fluid retention and anxiety.

### F.2.3. Prostaglandins

The prostaglandins, $PGE_2$ and $PGI_2$ (prostacyclin), have powerful vasodilatory and antiaggregatory effects. Prostacyclin and its stable analogue, iloprost, have proved successful in reducing the frequency and duration of vasospastic attacks in Raynaud's patients (Belch *et al.*, 1983; Yardumian *et al.*, 1988), and have been found to be effective in patients with secondary Raynaud's phenomenon who failed to respond to other forms of treatment (Watson & Belcher, 1991). Results from a study using $PGE_2$ are less convincing (Mohrland *et al.*, 1985). The mechanism of the prolonged improvement (several months) found in most studies after brief prostacyclin infusion is not understood; the vasodilatory and antiaggregatory effect declines shortly after treatment (Dowd *et al.*, 1982). Prostaglandin therapy can cause side effects such as headaches, flushing, diarrhoea, and postural hypotension, but a more serious criticism of this treatment has been raised by Kovacs and colleagues (1991), who report that patients receiving such therapy have a potential risk of thromboembolism. However, the methodology used by Kovacs *et al.* to measure coagulability has been questioned (Belch *et al.*, 1991). Until recently, this vasodilatory form of treatment had the disadvantage that it could only be given by intravenous infusion, as prostacyclin is easily destroyed in the gut. Although objective assessment of the effectiveness of oral iloprost failed to reach significance, a recent study has shown that Raynaud's patients found an subjective improvement in their symptoms (Belch *et al.*, 1995) which may make this therapy more readily available in the future, although at present it is usually confined to patients with severe secondary Raynaud's phenomenon due to the high incidence of side effects mentioned above. A transdermal $PGE_2$ preparation has also been developed, although it has since been withdrawn, despite promising effects in clinical trials (Belch *et al.*, 1985; Cooke *et al.*, 1985; Dunger *et al.*, 1985).

### F.2.4. Dietary fish oil & evening primrose oil

An alternative approach to prostacyclin therapy is to administer essential fatty acids, precursors of prostaglandins, in the form of dietary fish oil (DiGiacomo *et al.*, 1989) and evening primrose oil (Belch *et al.*, 1985), in order to stimulate the synthesis of endogenous prostaglandins. In addition to the resulting vasodilatation and inhibition of platelet aggregation, the fatty acids are thought to increase red blood cell deformability after becoming incorporated into their membrane (Green *et al.*, 1990). Interestingly, fish oil supplements were found to improve digital artery flow in primary, but not secondary Raynaud's patients (DiGiacomo *et al.*, 1989), although there is currently no evidence to confirm these early findings.

### F.2.5. ACE inhibitors

Inhibitors of the angiotensin converting enzyme (ACE)/kininase II inhibit the production of the vasoconstrictor, ANG II (AII) from ANG I, and the breakdown of the vasodilator, bradykinin. Their use in Raynaud's disease remains controversial. Uncontrolled studies, using captopril without a placebo, demonstated beneficial effects (Trubestein *et al.*, 1984; Tosi *et al.*, 1987) but, whilst the results of several controlled studies looked promising, they were inconclusive because statistical significance was not achieved (Miyazaki *et al.*, 1982; Janini *et al.*, 1988). Rustin and colleagues (1987) found no improvement of symptoms with captopril in patients with Raynaud's disease, and similar findings have been obtained with enalapril (Challenor *et al.*, 1991). Captopril may even induce digital vasospasm in patients being treated for hypertension (Havalka *et al.*, 1982), and in the study of Challenor and co-workers (1991), a substantial number of patients experienced more attacks during enalapril treatment.

### F.2.6. Miscellaneous drug treatments

#### F.2.6.1. $\alpha_1$-adrenoceptor antagonists

The use of selective $\alpha_1$-adrenergic antagonists, such as prazosin (Wollersheim *et al.*, 1986) and thymoxamine (Grigg *et al.*, 1989), can reduce vasospasm in some patients with Raynaud's disease but are associated with considerable side effects, including headaches, dizziness and palpitations - especially during exercise - though these effects are usually less severe with thymoxamine (Aylward *et al.*, 1982). Although it is the $\alpha_2$-adrenoceptors that have been implicated in the pathogenesis of Raynaud's disease (see above), selective $\alpha_1$-adrenoceptor antagonists are preferable to nonspecific $\alpha$-adrenoceptor blockers like phentolamine, since vasodilatation mediated by endothelial $\alpha_2$-adrenoceptors is not prevented, and the amount of noradrenaline released from nerve terminals is still regulated by the pre-synaptic negative-feedback $\alpha_2$-adrenoceptor.

#### F.2.6.2. Thromboxane synthetase inhibitors

Antiaggregatory drugs, such as dazoxiben, act by inhibiting thromboxane synthetase to prevent the production of $TXA_2$, a potent platelet aggregant and vasoconstrictor. In addition they increase prostacyclin formation by diverting the metabolism of $PGH_2$ exclusively down the prostacyclin synthetase pathway. Their vasodilatory action therefore prompted several investigators to study their effectiveness in Raynaud's disease (Jones & Hawkey, 1983; Coffman & Rasmussen, 1984; Luderer *et al.*, 1984). The results of these studies were disappointing; only small reductions in the frequency of attacks were observed, suggesting platelets are probably not playing a pathophysiological role in primary Raynaud's disease (see above).

#### F.2.6.3. Nitrovasodilators

The effects of topical glyceryl trinitrate (GTN) in Raynaud's disease are conflicting. Some trials have shown improvement (Nahir *et al.*, 1986), whilst others have not, despite subjective efficacy (Sovijarvi *et al.*, 1984; Teh *et al.*, 1995). Headaches and

dizziness, which are common complaints, are a major deterrent for its use. GTN has a greater vasodilator action on veins than on arteries; because the vasospasm in Raynaud's disease affects primarily the arterial circulation, higher doses of GTN are required to induce arterial vasodilatation, thus increasing the likelihood and severity of headaches.

### F.2.6.4. Fibrinolytics

Patients with primary and secondary Raynaud's phenomenon have been shown to have a decreased blood fibrinolytic activity, increased blood viscosity and increased plasma fibrinogen levels (Jarrett *et al.*, 1978). The fibrinolytic drug stanozolol, an anabolic steroid, has been examined in Raynaud's patients. In one study, 80% of patients reported a reduction in the frequency, severity and duration of their vasospastic attacks (Jarrett *et al.*, 1978). Jayson and colleagues (1991), however, failed to show any beneficial effects with stanozolol. Given that anabolic steroids can cause amenorrhoea and hirsutism, in addition to other side effects including oedema, acne, and potential liver damage, it is not wise to use these drugs in Raynaud's patients, the majority of whom are female.

### F.2.7. Behavioural treatment

Behavioural treatment in Raynaud's disease aims to give patients self-control of their peripheral blood flow. Temperature biofeedback, conditioning or relaxation techniques (autogenic training) are the commonest behavioral approaches used (Freedman, 1989). Although positive results have been shown by some investigators (Freedman *et al.*, 1981; Jobe *et al.*, 1982; Yocum *et al.*, 1985), behavioral treatments require a lot of time, effort and motovation from the patient and their physician, and few studies have examined the long term success of this form of therapy.

### F.2.8. Sympathectomy

Cervical sympathectomy has not been found to prevent Raynaud's disease affecting the hands; any reported improvements often disappear shortly after the operation, with vasospastic attacks recurring within 6 months to 2 years (Johnston *et al.*, 1965). In Raynaud's disease which affects the toes, however, lumbar sympathectomy has proved more successful in preventing vasospasm, and the results are often permanent (Janoff *et al.*, 1985).

### F.2.9. Plasmapheresis

Plasmapheresis, or plasma exchange, is an expensive procedure which has been shown to reduce blood viscosity (Talpos *et al.*, 1978), and is reserved for patients with severe Raynaud's phenomenon with digital ulcers, who are unresponsive to other forms of treatment.

### F.2.10. Future developments

It has been shown that the digital skin of patients with Raynaud's disease has a deficiency in calcitonin gene-related peptide (CGRP)-containing neurons (see above). The use of CGRP in the treatment of Raynaud's disease looks promising: in one study, intravenous infusion of CGRP into Raynaud's patients increased hand-skin blood flow throughout the infusion period, in contrast to prostacyclin, and its effects persisted for 3 days after the infusion (Shawket *et al.*, 1991). Similarly, Bunker and colleagues (1993) found intravenous CGRP to be an effective peripheral cutaneous vasodilator in patients with severe Raynaud's phenomenon. However, because intravenous CGRP treatment can only be administered in hospital, it is likely to be reserved for those patients suffering from severe Raynaud's phenomenon. The development of non-peptidergic CGRP agonists would enable wider use of this form of treatment.

Flavonoids and ginkgolides extracted from the leaves of the *Ginkgo biloba* tree have a potential role in the treatment of Raynaud's disease. Among its known effects, ginkgo extract has been shown to cause an increase in blood flow and a decrease in blood viscosity (Kleijnen & Knipschild, 1992). This may be due to its antagonistic effects on platelet activating factor (PAF) or its ability to act as a superoxide anion scavenger, thereby increasing the half-life of NO (Robak & Gryglewski, 1988).

Whilst the therapies described above can be useful in some patients with Raynaud's, none are effective in all cases. Clearly there is a need to develop specific and effective treatments for Raynaud's disease, but until the exact cause of the disorder is identified, therapy has to remain on a 'trial and error' basis. A clearer understanding of the mechanisms underlying vasospasm may also indicate new directions for the treatment of migraine, variant angina and ocular vasospasm, three vasospastic conditions with which Raynaud's disease is associated.

## G. Aims

The object of this thesis was to examine the role of the vascular endothelium in the development of cold-induced vasoconstriction, and in the pathophysiology of vasospasm in Raynaud's disease. From a review of the literature at the outset of this thesis it was hypothesised that, firstly, in healthy subjects during cold exposure, vasoconstriction to ET is opposed by increased NO production, the balance favouring vasoconstriction but not of sufficient severity or duration to cause vasospasm. Secondly, the 'local fault' in Raynaud's disease is within the vascular endothelium, and is associated with an imbalance between the production of ET and NO, favouring prolonged vasospasm, probably through a mechanism involving impaired NO synthesis.

The specific aims arising from the above hypotheses were:

1. To investigate the effect of cooling on the contractile response to ET-1 in resistance arteries obtained from rat and human tissue.

2. To determine whether cooling exerts a general effect on the contractile response of the vascular smooth muscle by studying its effects on the $\alpha_1$-adrenoceptor agonist, phenylephrine, and the direct activator of smooth muscle, potassium chloride.

3. To assess the contribution of the vascular endothelium to any cold-induced effects on the above vasoconstrictor substances by examining the effects of endothelial removal, inhibitors of the NO and $PGI_2$ pathways, and of ET-receptor antagonists.

4. To investigate the effect of cooling on vasorelaxation to the endothelium-dependent dilator, acetylcholine (ACh), and the endothelium-independent dilator, sodium nitroprusside (SNP), in resistance arteries obtained from rat and human tissue.

5. To investigate potential mediators of cold-induced vasoconstriction *in vivo* using the autoperfused hindlimb of the anaesthetised rat.

6. To compare the responses to ET-1, ACh and SNP in resistance arteries obtained from subcutaneous fat biopsies from patients with Raynaud's disease and healthy control subjects, in order to determine the potential contribution of the endothelium in the pathogenesis of Raynaud's disease.

# CHAPTER 2:
# MATERIALS AND METHODS

## 2.1. Small vessel arteriograph (perfusion myograph)

### 2.1.1. Introduction

Prior to the 1970's, experimental work on isolated vessels had been mostly confined to large arteries, the aorta in particular. However, it was recognised that most of peripheral vascular resistance lay in the smaller arterial vessels, the so-called 'resistance' arteries, which are chiefly responsible for regulating blood flow and capillary pressure (Furness & Marshall, 1974). In 1976 Mulvany and Halpern published a study of resistance arteries using the then new technique of wire myography (Mulvany & Halpern, 1976), first proposed by Bevan & Osher (1972). Essentially, the wire myograph involves mounting the microvessel as a ring preparation by passing two fine wires through the lumen. Wall tension is then measured by recording the isometric force exerted on the wires. Since its introduction, the wire myograph has been modified and improved to produce a more physiological method of studying microvessels. These modifications have essentially allowed a pressure to be applied across the vessel walls (Duling *et al.*, 1981; Sipkema & Westerhof, 1989; Halpern & Kelley, 1991; Hoogerwerf *et al.*, 1992). The small vessel arteriograph, or perfusion myograph, used in the experimental procedures during this thesis was the version developed by William Halpern and colleagues (Halpern & Kelley, 1991), and manufactured by Living Systems Instrumentation Inc., Burlington, Vermont, USA.

There are several advantages in using the perfusion myograph rather than the wire myograph; these allow the vessels under study to be closer to physiological conditions:

- the axial length can be adjusted to compensate for retraction of arteries after dissection (but the set length can only approximate that found *in vivo*)
- a transmural pressure exists across the vessel wall
- because the vessel is pressurised, it assumes a more normal, cylindrical shape than in the wire myograph, where it is pulled out flat

- the endothelium is untouched along the area under study, whereas wires impinge, and cause local damage to the endothelium in the wire myograph
- the diameter is allowed to change when the vessel contracts and relaxes
- drugs can be independently administered both intra- and extra-lumenally

Buus and co-workers (1994) have demonstrated significant differences in the responsiveness of small arteries between the wire and pressure myograph; the sensitivity to α-adrenoceptor agonists was greater in arteries mounted in the pressure myograph. The authors suggest the responses found in the pressure myograph may be of greater relevance to the situation *in vivo*, compared to those found with wire myography.

### 2.1.2. Isolation of microvessels

#### 2.1.2.1. *Rat mesenteric vessels*

Male Wistar rats were obtained from in-house stock bred at the Biomedical Research Facility (Western General Hospital, Edinburgh) and maintained on standard chow and tap water *ad libitum*. The animals (11-16 weeks of age) were killed by cervical dislocation and a ventral midline incision was made. Mesenteric vascular beds were removed and pinned out in a silicone-coated (Sylgard, Dow-Corning, U.K.) dissecting dish containing physiological salt solution (PSS) (mM: 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 $KH_2PO_4$, 25.0 $NaHCO_3$, 0.026 EDTA, and 5.5 glucose) at room temperature (22 - 24°C).

For resistance arteries of ~200-300μm in diameter, second and third order vessels, i.e those appearing after the 2nd and 3rd branch of the superior mesenteric artery (Figure 2.1), were excised under a dissection microscope (Zeiss, U.K.) using No.5 watchmaker forceps and fine ocular scissors (Altomed Ltd, Tyne & Wear, U.K.). Care was taken during the dissection not to touch the section of vessel to be studied. To avoid touching the vessel, surrounding fat was gently pulled away from it to

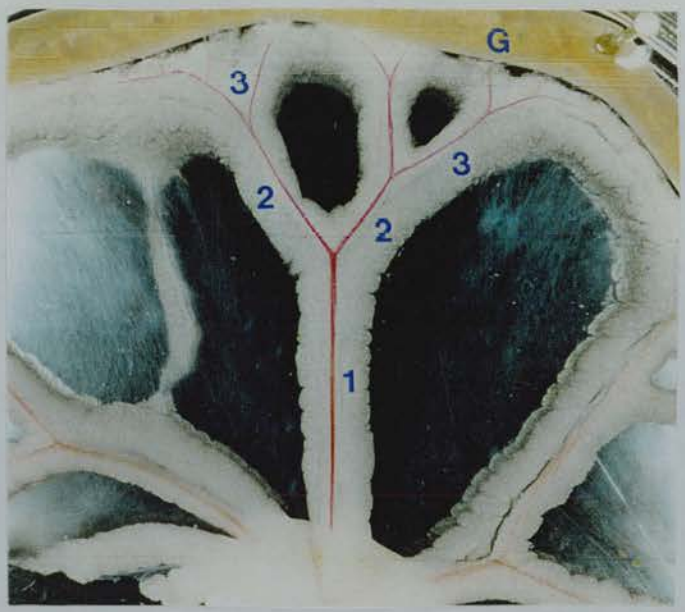



***Figure 2.1.*** *Photograph of an excised rat mesenteric bed pinned out on a silicone-coated dissecting dish, showing the location of 1st (1), 2nd (2) and 3rd (3) order mesenteric arteries. G = gut wall. (x3 magnification).*

expose the thin membrane running between the fat and the artery. This membrane was cut, thus isolating the artery from the fat cells. The artery was then excised, with a diagonal cut being made to the proximal end. It was important to be able to differentiate the two ends of the artery in order to mount it in the correct orientation (proximal -> distal) in the myograph, i.e. any flow introduced through the lumen would have been in the same direction as that of blood *in vivo*.

#### 2.1.2.2. *Human vessels: tissue from surgery*

The use of human tissue in the following studies was approved by the Lothian Research Ethics Committee. Subcutaneous fat biopsies obtained from 15 patients (age range 18-77; 11 males, 4 females; Table 2.1) undergoing elective gastro-intestinal surgery at the Western General Hospital, were placed directly into PSS. Quite often, due to the time-course, samples were kept in a refrigerator, at 4°C, overnight before beginning the dissection procedure the following day. This does not appear to have any adverse effects on the vessels (Laing *et al.*, 1995). Overnight storage of vessels may allow any depressant effects of the general anaesthetic to wear off, as volatile anaesthetics have been shown to inhibit endothelium-dependent dilatation (Muldoon *et al.*, 1988; Johns, 1989; Stone & Johns, 1989); it was therefore important to

randomise the order of experiments so that the anaesthetic did not influence one particular group over another. Small arteries (~300μm) were dissected out from the biopsy in a similar way to that described for rat mesenteric arteries. Unless a branch point existed in the vasculature, it was hard to identify the direction the artery was orientated. This meant some vessels were probably mounted in the opposite direction from that *in vivo*. The danger of mounting vessels in such a way is that the endothelium is at risk of damage or removal. This did not pose a serious problem though, because the only vessels which were exposed to any substantial amount of flow through their lumen, were those which were to have their endothelium removed intentionally by air (see later).

***TABLE 2.1.*** *Patient details for vessels studied at 37°C and 24°C*

| | 37°C | 24°C |
|---|---|---|
| Age (years) | 58 ± 6 | 57 ± 10 |
| Sex ratio (M:F) | 6 : 2 | 5 : 2 |

| Operation performed: | | |
|---|---|---|
| Hemicolectomy | 3 | 1 |
| Inguinal hernia repair | 3 | 2 |
| Resection of rectum | 1 | 1 |
| Gastropexy | 1 | - |
| Prostatectomy | - | 1 |
| Splenectomy | - | 1 |
| Hartman's operation | - | 1 |

*Values are mean ± SEM for n = 8 at 37°C and n= 7 at 24°C.*

### 2.1.2.3. *Human vessels: tissue from gluteal fat biopsies*

Thirty-nine volunteer control subjects and Raynaud's patients were recruited in this study (controls: 29-64 years; 14 females and 4 males. Raynaud's patients: 28-62 years; 18 females and 3 males) (see Table 2.2). Patients with primary Raynaud's disease were classified according to the criteria of Allen & Brown (1932): (i) colour

changes occurring bilaterally in the hands evoked by cold or emotional stimuli; (ii) absence of gangrene; (iii) absence of any known causative disease; and (iv) history of symptoms for a minimum of two years. The mean duration of the Raynaud's disease was 18 ± 3 years. One patient had Raynaud's phenomenon secondary to Sjögren's syndrome. All subjects had an alcohol intake <14 and <21 units/week, for females and males respectively, and were normotensive (systolic blood pressure <140 mmHg; diastolic blood pressure <90 mmHg). Three control subjects, and one Raynaud's patient were smokers.

The protocol of this study was approved by the Lothian Research Ethics Committee, and written, witnessed, informed consent was obtained from each subject. All subjects abstained from aspirin-containing drugs for one week, and from caffeine-containing drinks or alcohol for 12 hours before the biopsy was taken. A 50ml blood sample was taken from each subject for immunological screening and for the measurement of haemoglobin, erythrocyte sedimentation rate, urea and electrolytes, glucose and creatinine.

***TABLE 2.2.*** *Subject and patient details for vessels studied at 37°C and 24°C*

| | Control subjects | | Raynaud's patients | |
|---|---|---|---|---|
| | 37°C | 24°C | 37°C | 24°C |
| Age (years) | 42 ± 4 | 37 ± 3 | 47 ± 2 * | 45 ± 3 |
| Sex ratio (M:F) | 1 : 7 | 2 : 6 | 2 : 10 | 1 : 9 |

*Values are mean ± SEM. *P<0.05 compared to control group at 24°C (unpaired t-test). Note: the details refer only to the volunteers whose biopsies yielded arteries which were used in the study (see below).*

Skin biopsies, approximately 2 cm long, 0.75 cm wide and 0.75 cm deep, were taken from the gluteal region under local anaesthetic (1% lignocaine, Astra Pharmaceuticals Ltd., U.K.) (Aalkjaer *et al.*, 1987) by Dr Charles J. Ferro at the Clinical Pharmacology Unit and Research Centre, Western General Hospital, and were placed directly into PSS. Small arteries (~300μm) were dissected out from the biopsy as

described above. From the 39 biopsies taken, a total of 34 yielded arteries, thus making an 87% success rate of obtaining arteries from the biopsies. Usually, the biopsy yielded more than one artery. If there were two arteries, the first was mounted in the myograph, whilst the second was kept in a refrigerator overnight to use the following day. The order of experiments performed on the vessels was randomised to prevent any influence of overnight storage on the results.

### 2.1.3. Mounting and pressurising of vessels in myograph

Once dissected, arteries were carefully transferred on the tip of a pair of forceps to the vessel chamber of the myograph (Living Systems Instrumentation, Burlington, Vermont, U.S.A.), which contained 10ml of PSS. Here, the resistance arteries were tied onto two fine glass cannulae (~150μm tip diameter) using single-fibre silk threads. This was done by firstly gently pulling the proximal end of the vessel onto the cannula tip until approximately 200μm of the tip was inserted into the lumen of the vessel. Two silk threads (~20μm diameter) which had already been looped onto the cannula, were then slid down over the vessel and secured by pulling both ends with forceps (Figure 2.2a). To remove any blood present in the lumen of the artery, which would obstruct measurement of the diameter, the stopcock to the proximal cannula was opened and a slow flow of PSS was passed through the lumen, by means of a miniature peristaltic pump (PS/200, Living Systems Instrumentation Inc., Burlington, Vermont, USA). Care was taken not to raise the intra-luminal pressure above 3-4 mmHg at this stage, in order to avoid damage to the endothelium. Once the lumen was cleared of blood, the stopcock was closed and the distal end of the artery was then secured onto the distal cannula in the same way as described for the proximal end. Each end of the vessel had two ties therefore, which helped to ensure it would maintain a set pressure.

An intraluminal pressure of 60 mmHg for rat, and 50 mmHg for human vessels, was achieved by slowly introducing PSS through the vessel lumen using the miniature

peristaltic pump, which was connected to a pressure servo unit (Figure 2.2b). As the vessels were pressurised, they usually developed a bend due to a change in axial length. These 'buckles' were removed by gently moving back the proximal cannula using the attached length transducer, so as to reset the vessel to its 'original' axial length (prior to dissection), whilst being careful to avoid introducing any axial stretch. Once the pressure had reached the desired level set on the servo unit, the artery was checked for leaks by changing the pressure servo from automatic to manual mode (pump no longer running). If the mounted arteries had no leaks, the pressure reading remained at the original setting. If a fall in pressure was seen, either a further attempt was made at securing the ties, or the artery was discarded and another one mounted. The pressure servo unit maintained the set intraluminal pressure throughout the experiment. Pressures of 60 mmHg for rat, and 50 mmHg for human vessels, were chosen because arteries of the size used in these experiments have been estimated to experience pressures approximately 50% of mean arterial pressure *in vivo* (Halpern & Kelly, 1991).

The myograph was placed on an inverted stage microscope (Nikon TMS-F, Japan) which was connected to a monochrome television camera (Burle, U.S.A.), thus allowing the mounted arteries to be visualised on a television monitor (Burle, U.S.A.) (Figure 2.3). The lumen diameter and wall thicknesses were measured using a video dimension analyser (Living Systems Instrumentation, Burlington, Vermont, U.S.A.), which had been calibrated against a stage micrometer (resolution = 1μm). The video dimension analyser is able to detect changes in optical density along a chosen scan line. The walls have a much higher optical density than the rest of the vessel, hence continuous measurements of both wall thicknesses and lumen diameter can be made.

Once mounted, the arteries were continously superfused with PSS which was gassed with 95% $O_2$ and 5% $CO_2$. The temperature of the fluid within the vessel chamber, measured using a digital thermometer (TM-903, Lutron Equipment, UK) held in close

***Figure 2.2a.*** *Photograph of the myograph vessel chamber where the proximal end of a resistance artery (~250 μm i.d.) has been secured onto the first glass cannula using single fibre silk thread (x5 magnification).*

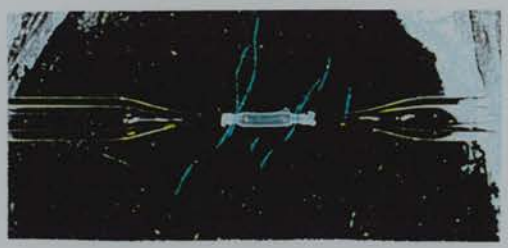


***Figure 2.2b.*** *After securing the distal end of the resistance artery to the second glass cannula, an intraluminal pressure of 60mmHg has been introduced by means of a miniature peristaltic pump and pressure servo control unit (x5 magnification).*

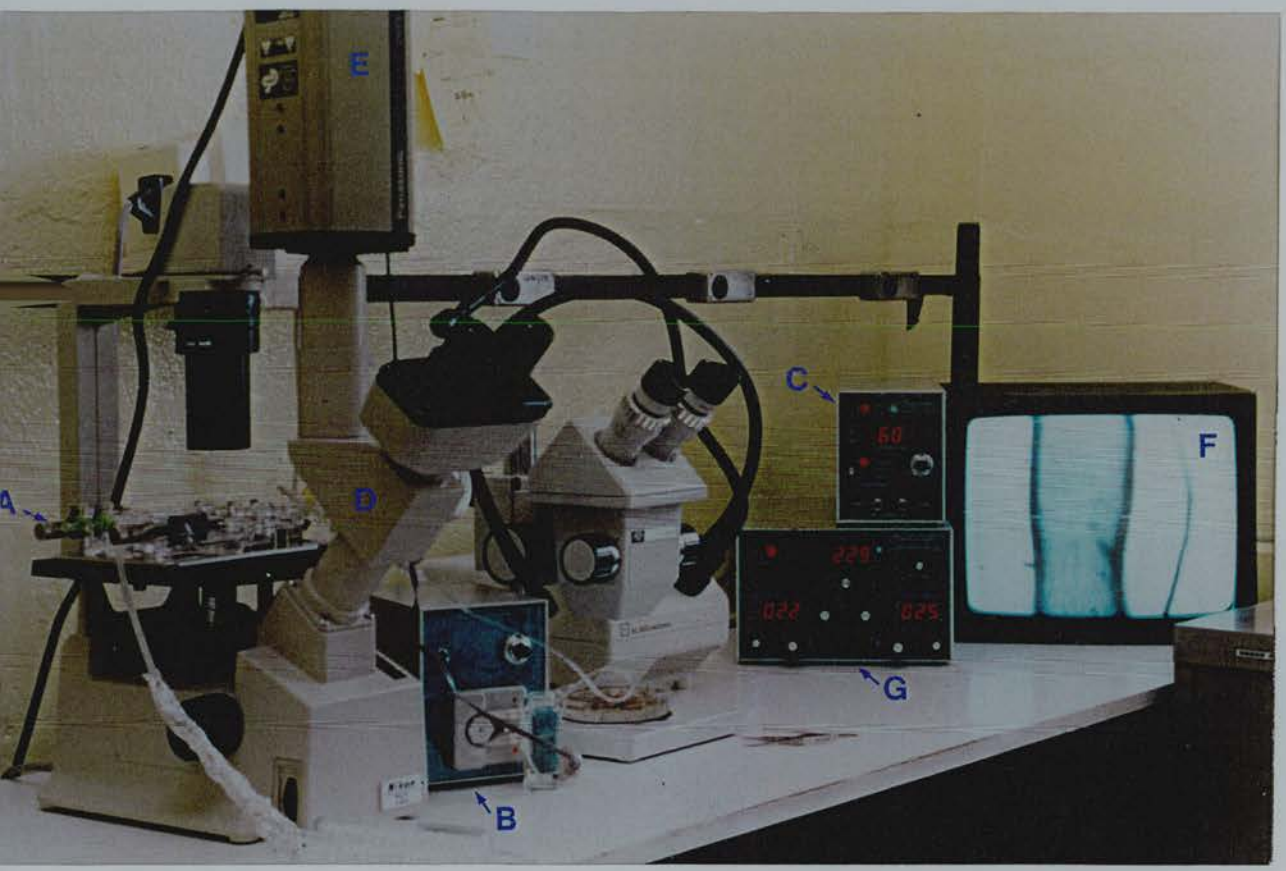



***Figure 2.2.*** *Photograph showing the components of the small vessel arteriograph (perfusion myograph): vessel chamber (A); miniature peristaltic pump (B); pressure servo unit (C); inverted stage microscope (D); television camera; (E); television monitor (F); and video dimension analyser (G).*

proximity to the vessel, was maintained at 37°C; before entering the vessel chamber the superfusate was passed through a glass heat-exchanger, which was warmed with circulating water from a water bath (at 40°C), and a Peltier heat-exchange unit (MHE-1, Moor Instruments, Millwey, Axminster, Devon, UK).

### 2.1.4. Cooling procedure

Cooling of the arteries to 24°C was achieved by firstly switching off the water circuit to the glass heat-exchanger, and then re-setting the Peltier unit from 37°C to 27°C; because of an overshoot of temperature during cooling, a temperature higher than 24°C was chosen to avoid cooling to below 24°C. Once the temperature stabilised at around 24°C, after approximately 5 minutes, fine tuning of the Peltier unit maintained a constant temperature of 24°C.

### 2.1.5. De-endothelialisation

In large arteries the most common approach for removing the endothelium is by mechanical means, usually by rubbing the inner surface of a vessel segment with a small stick or wire. This technique is difficult to apply to small resistance arteries because of their fragility. A wide range of alternative chemical and enzymatic methods have been employed to remove or inactivate the endothelium from microvessels; for example, perfusion with detergents such as 3-[(3-cholamidopropyl)dimethyl ammonio]-1-propane sulphonate (CHAPS) (Hiley *et al.*, 1987) or sodium deoxycholate (Byfield *et al.*, 1986), dissolving the intracellular matrix with enzymes such as collagenase (Carvalho & Furchgott, 1981) or elastase (Furchgott & Zawadzki, 1980), rupturing endothelial cells osmotically with distilled water (Criscione *et al.*, 1984), or perfusion with 40mM potassium chloride (Griffith *et al.*, 1985). Unfortunately, chemical techniques are difficult to control precisely (e.g. exposure time, shear rate) and carry the risk of damaging adjacent smooth muscle cells. It then becomes difficult to know with certainty whether an observed difference in the vessel response is due specifically to endothelial cell removal.

Alternative ways of denuding resistance arteries mechanically are the introduction of a single hair into the vessel lumen (Osol *et al.*, 1989), and perfusion of the lumen with an air bubble (Ralevic *et al.*, 1989; Bjorling *et al.*, 1992; Falloon *et al.*, 1993). The 'air bubble technique' is employed by Professor Heagerty's group in Manchester, and was the chosen method here, as it gave consistent results during preliminary tests.

### 2.1.5.1. Air bubble technique

Removal of the endothelium was achieved by passing air through the lumen of the mounted artery, similar to the method described by Falloon *et al.* (1993) as follows: first, the setting on the length transducer was recorded so that the vessel could later be reset to its original axial length. The pressure within the vessel was then slowly reduced whilst the axial length was simultaneously re-adjusted to prevent any axial stretch on the vessel. The stopcock at the end of the distal cannula was then opened, and after disconnecting the luer fitting at the proximal end of the vessel chamber, an air bubble about an inch in length was introduced into the tubing (Tygon: I.D. 1/32") feeding the proximal cannula. This was achieved by switching to the flow mode on the pressure-servo box whilst reconnecting the luer fitting. The air was slowly moved down the tubing to the vessel at a flow rate which produced a pressure of 20-30 mmHg. A series of small air bubbles (usually between 10 and 15) were passed through the lumen of the vessel. Once all of the air had passed through the vessel, flow was maintained for a further 2-3 minutes to allow endothelial debris to be washed away. Switching back to the pressure mode on the pressure servo unit, the stopcock was closed again and the vessel restored to its original axial length and pressure.

Removal of the endothelium was assessed using various methods. In every vessel studied, functional tests were made using the endothelium-dependent vasodilator, acetylcholine (ACh, $10^{-6}$ to $10^{-4}$M), administered during contraction to phenylephrine ($\alpha_1$-adrenoceptor agonist, PE, $10^{-5}$M) (see Experimental protocol below). Some

vessels were studied morphologically by means of scanning and transmission electron microscopy, or by confocal microscopy.

### 2.1.5.2. Confocal microscopy

Four vessels (from 1 rat) were mounted in the myograph and contractility assessed with PE. The first two arteries were gently removed from the cannulae and immersed in the nuclear fluorescent dye, ethidium bromide (5μg/ml), for 30 minutes. The third and fourth arteries were denuded (confirmed by loss of response to ACh) using the 'air bubble technique' before removal from the cannulae and ethidium bromide staining. The vessels were then viewed under a confocal microscope (Odyssey XL, Noran Instruments Inc., U.S.A) at the Department of Physiology, University of Glasgow, with the assistance of Mr Craig Daly and Dr Silvia Arribas. Essentially, the confocal microscope allows 3D images to be visualised by reconstructing a series of 2D confocal images. It was used here simply to scan the vessels at different depths in order to detect the absence or presence of the endothelium. Because ethidium bromide labels the nuclei of cells which have a permeabilised membrane, i.e. those which are damaged, it is possible to visualise the degree of damage, if any, caused to endothelial and smooth muscle cells after the passage of an air bubble through the lumen.

### 2.1.5.3. Fixation of vessels for electron microscopy

Arteries to be examined by electron microscopy were firstly fixed. Three vessels (from 2 rats) which had not been mounted on the myograph were fixed immediately after dissection. Two vessels (from 2 rats) were mounted in the myograph and contractility assessed with PE. After removal from the myograph they were fixed. Another three vessels (from 2 rats) were mounted and denuded using the 'air bubble technique', confirmed with acetylcholine, before removal and fixation. The vessels were fixed by immersion into Karnovsky's fixative (see section 2.4.) for exactly 2 hours in a refrigerator, and were then washed in fixative free buffer and stored in the fridge overnight, before being sent in a vacuum flask containing ice to Dr. K. Cracknell at

the Division of Physiology, St Thomas' Hospital in London for further processing. The following procedures relating to electron microscopy were carried out by Dr Cracknell and colleagues in London.

#### 2.1.5.4. Transmission electron microscopy

Arteries were rinsed in 0.1M physiological buffer and fixed with 1% osmium tetrooxide. The samples were dehydrated in a graduated series of ethanol solutions followed by propylene oxide, before being embedded in Araldite Resin (Fisons, Loughborough, U.K.). Transverse sections (~90nm thick) were cut using an LKB ultratome III microtome. After staining with uranyl acetate and lead citrate, the vessels were examined on a Zeiss EM 10C electron microscope.

#### 2.1.5.5. Scanning electron microscopy

Arteries were dehydrated in ethanol as above, then dried using a critical point drier (Fisons, Loughborough, U.K.). The samples were then mounted on a scanning electron microscopy stub, endothelial layer uppermost, using 'sticky tabs' (Agar scientific, Stansted, U.K.), after which they were sputter coated (Polaron SEM coating unit E5000) with gold (Agar scientific, Stansted, U.K.) and viewed in a Hitachi S-510 scanning electron microscope.

### 2.1.6. Experimental protocol

#### 2.1.6.1. *Rat Vessels*

Following a 60-90 min equilibration, during which the temperature of the vessel chamber was raised from room temperature to 37°C, three 'wake-up' doses of PE ($10^{-5}$M) were given to produce a contraction to < 35% of resting lumen diameter. ACh ($10^{-6}$M) was given during the third contraction to assess endothelial integrity.

#### 2.1.6.2. *Human Vessels*

Following a 60-90 min equilibration, during which the vessel chamber was superfused with PSS, continuously gassed with 95% $0_2$:5% $CO_2$, and the temperature

raised to 37°C, the contractility of the arteries was assessed separately using PE ($10^{-5}$M, Sigma) and potassium chloride (KCl, 60mM, Sigma). KCl was given in addition to PE because, unlike rat vessels, human arteries did not always respond to the $\alpha_1$-adrenoceptor agonist, implying there was a reduced density and/or sensitivity of these receptors in the unresponsive human vessels, compared to arteries from the rat. ACh ($10^{-6}$M, Sigma) was given during maximum contraction to PE to assess endothelial integrity.

Removal of the endothelium was achieved by passing air through the lumen of the vessel (see above) and confirmed functionally by the loss of ACh-induced (up to $10^{-4}$M) relaxation during constriction to a fourth dose of PE. Cooling was achieved by passing the superfusate through a Peltier heat-exchange unit (see above) before it entered the arteriograph.

Cumulative concentration-response curves were generated using a reperfusion circuit. Because drugs such as ET-1 are expensive, other users of the myograph have simply added drugs directly into the vessel chamber, during which the superfusate is switched off. Although this method minimises the amount of drug used, it has the disadvantage that the temperature is not maintained at 37°C. From Figure 2.4, it can be seen that switching off the superfusate for the approximate time required to complete a concentration-response curve to ET-1 (~40 min.), results in a rapid and sustained decline of the chamber temperature. Since the effects of cooling are the focus of this thesis, this is clearly unsatisfactory.

The reperfusion circuit was set up in order to obtain cumulative concentration-response curves whilst maintaining a constant temperature in the vessel chamber. The total volume of the circuit was 30 mls, as opposed to just 10 ml in the vessel chamber; thus three times as much drug had to be used with the reperfusion method. This did not pose a serious problem however, since the maximum response to all the agonists used in this thesis was easily obtainable with the final concentrations achieved in the vessel chamber.

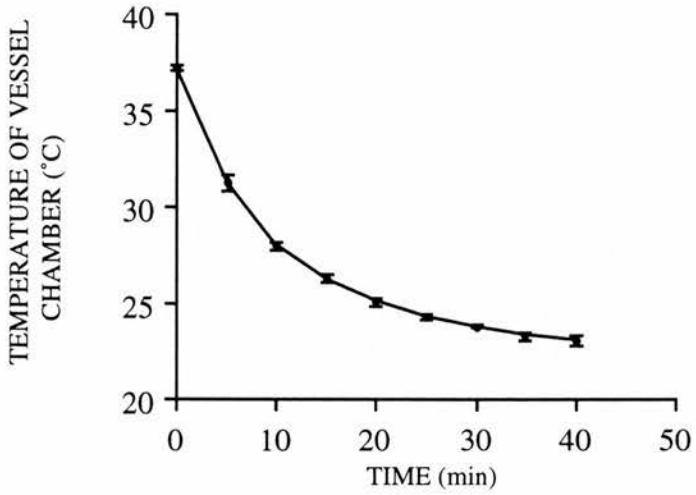



***Figure 2.4.*** *The effect of switching off the superfusion circuit on vessel chamber temperature over a 40 min period. Mean ± SEM of n=3.*

### 2.1.6.3. An investigation of vasoconstrictor substances

Cumulative concentration-response curves to the agonists shown in Tables 2.3 and 2.4. were obtained at 37°C or 24°C, and with endothelium either intact or removed, in a randomised order. In further experiments, the effects of the nitric oxide synthase inhibitor, $N^G$-nitro-L-arginine methyl ester (L-NAME; $10^{-4}$M), the cyclooxygenase inhibitor, indomethacin ($10^{-5}$M), or the $ET_{A/B}$ receptor antagonist, bosentan ($10^{-6}$M), were examined (Tables 2.3 and 2.4).

### 2.1.6.4. An investigation of vasodilator substances

Cumulative relaxation-response curves to the endothelium-dependent dilator, ACh, and the endothelium-independent dilator, sodium nitroprusside (SNP), were obtained at 37°C or 24°C, and with endothelium either intact or removed (Table 2.5). The endothelium was removed in the experiments involving SNP in an attempt to standardise conditions, because basal production of NO can affect the responses to exogenous nitrovasodilators (Moncada *et al.*, 1991).

| | **ET-1** ($10^{-12}$ - $3x10^{-7}$M) 37°C & 24°C | **PE** ($10^{-8}$ - $10^{-5}$M) 37°C & 24°C | **KCl** (10 - 60mM) 37°C & 24°C |
|---|---|---|---|
| Intact | ✓ | ✓ | ✓ |
| Denuded | ✓ | ✓ | ✓ |
| L-NAME / Indo. | ✓ | ✗ | ✓ |
| Bosentan | ✓ | ✓ | ✗ |

***Table 2.3.*** *Concentration-response curves to agonists generated in rat mesenteric resistance arteries. Note: for potassium chloride (KCl), combined $N^G$-nitro-L-arginine methyl ester (L-NAME)/indomethacin (indo.) was used, whereas the effects of separate L-NAME and indomethacin administration was studied for endothelin-1 (ET-1). (See text for dose of inhibitor/antagonist used).*

| | **Surgical biopsy** **ET-1** 37°C & 24°C | **Gluteal biopsy** **ET-1** 37°C & 24°C |
|---|---|---|
| Intact | ✓ | ✓ |
| Denuded | ✗ | ✓ |

***Table 2.4.*** *Concentration-response curves to endothelin-1 (ET-1) ($10^{-12}$ - $3x10^{-7}$M) generated in human resistance arteries obtained from surgery and from gluteal biopsies.*

| | **ACh** ($10^{-10}$ - $10^{-4}$M) 37°C & 24°C | **SNP** ($10^{-10}$ - $10^{-3}$M) 37°C & 24°C |
|---|---|---|
| Intact | ✓ | ✗ |
| Denuded | ✗ | ✓ |

***Table 2.5.*** *Concentration-relaxation curves to vasodilators generated in rat mesenteric resistance arteries and human resistance arteries obtained from gluteal fat biopsies.*

### 2.1.7. Statistical Analysis

Responses are expressed as a percentage (%) of the contraction to PE (rat) or KCl (human) at 37°C. $EC_{50}$ values were calculated by plotting individual concentration-response curves (Cricket-graph, Macintosh) and measuring, by hand, the concentration of the agonist producing 50% of the maximum contraction of each individual curve. $EC_{50}$ and $E_{max}$ (the maximal contraction) values are shown as mean ± standard error of the mean (SEM), and n = the number of vessels studied. In cases where more than one vessel was obtained from one animal/biopsy, n/n = the number of vessels from the number of animals/patients studied (where appropriate). Note: the same experiments were never repeated in subsequent vessels from any one rat or biopsy tissue, i.e. subsequent vessels were placed in a different group to that of the first studied, thus n/n is only referred to when discussing combined data. Student's paired *t* test was used to compare the responses between 37°C and 24°C groups, intact and denuded groups, or absence and presence of antagonist groups. The significance of the data obtained from the human resistance artery studies was further analysed by two-way analysis of variance (ANOVA). Statistical significance was accepted when $P < 0.05$.

## 2.2. Autoperfused hindlimb of the anaesthetised rat

The rat autoperfused hindlimb preparation was set up with the aim of examining the effects of temperature on vascular resistance *in vivo*, and to characterise possible mediators of the responses using selective antagonists and inhibitors. It was anticipated that the results obtained from the physiological responses to cooling in the rat hindlimb would help to elucidate the mechanisms underlying the pathophysiology of Raynaud's disease.

The rat autoperfused hindlimb preparation was set up as a modification of the method used by Born & Palinski (1989); instead of perfusing the right hindlimb with blood from the carotid artery, the present technique used blood from the left femoral artery.

Before entering the right femoral artery, the blood was cooled using a rapid heat-exchange device, or Peltier cell, which allowed temperature changes to be made in a matter of seconds. It can be argued that this preparation is not representative of the conditions occuring in Raynaud's Disease, whereby it is the external surface of the limb, and not the blood, which is cooled initially. However, it would be difficult to measure the temperature the vasculature was subjected to if the hindlimb was cooled externally. An advantage of cooling the blood by means of the Peltier cell, is that the temperature of the blood going to the hindlimb can be monitored accurately, by means of a fine thermistor probe placed at the site where blood enters the hindlimb, and is reproducible from animal to animal.

### 2.2.1. General surgery

Male Wistar rats were obtained from Charles River (Kent, UK) and maintained on standard chow and tap water *ad libitum*. The animals (378 ± 9g; n=64) were anaesthetised with sodium pentobarbitone (60mg/kg i.p., Sagatal, Rhone Merieux Ltd., UK). A tracheal cannula was inserted to allow measurement of airflow using a pneumotachograph (CS5, Mercury Electronics, UK). Catheters filled with heparinised saline (50 units/ml) were inserted into the right jugular vein and right carotid artery for drug administration and blood pressure measurement by a pressure transducer (P23Dc, Statham, UK) respectively.

The rats were then heparinised (1000U/kg intra-venously i.v.) prior to cannulation of the right and left femoral arteries to prevent coagulation of blood in the perfusion circuit. Blood from the cannulated left femoral artery was passed into the right femoral artery using a peristaltic pump (MRHE 200, Watson Marlow, UK). A pressure transducer (P23Dc, Statham, UK), situated between the pump and the right hindlimb, measured hindlimb perfusion pressure (HLPP), which was used as an index of total vascular resistance of the hindlimb (Figure 2.5). The pump was set at a constant flow rate so that the HLPP matched the mean arterial blood pressure (MAP).

Rectal temperature was measured using a thermistor probe and maintained at 37±1°C by a homeothermic blanket connected to the probe (Harvard Apparatus Ltd., Kent, UK). Blood samples were taken approximately every hour from the carotid artery to monitor blood gas tensions and pH, using a pH/blood gas analyser (238, Ciba-Corning, UK).

### 2.2.2. Cold-induced vasoconstriction

Cooling was achieved by passing blood through a rapid heat-exchange element (Peltier cell) before it entered the right hindlimb (Figure 2.5). Initially, the effect of cooling on perfusion pressure was examined by changing the temperature from 37°C to 21°C in 4C° steps, each held for 2 minutes, before rewarming to 37°C in 4C° steps. Random temperature changes were also made in order to determine how reproducible the temperature-HLPP curves were (see Chapter 3). The reduction in temperature was associated with a rise in HLPP, implying cold-induced vasoconstriction was occurring. Temperature-HLPP curves were obtained before and after drug administration by cooling the blood in the 4C° steps outlined above.

In order to establish whether the rise in perfusion pressure seen during cooling was due to vasoconstriction of the hindlimb vasculature and not merely the result of a temperature-induced increase in blood viscosity, and hence an increase in resistance, the hindlimb was perfused with saline by removing the cannula from the left femoral artery and placing it into a vial containing saline (n=4). Only a single temperature-HLPP curve was obtained in any one animal during saline-perfusion, because the increase in blood volume following the perfusion would affect subsequent responses (see Chapter 3).

In four experiments, the skin from the hindlimb was surgically removed and temperature-HLPP curves carried out to determine what proportion, if any, of the rise in HLPP with cooling was mediated by the cutaneous circulation (see Chapter 3).

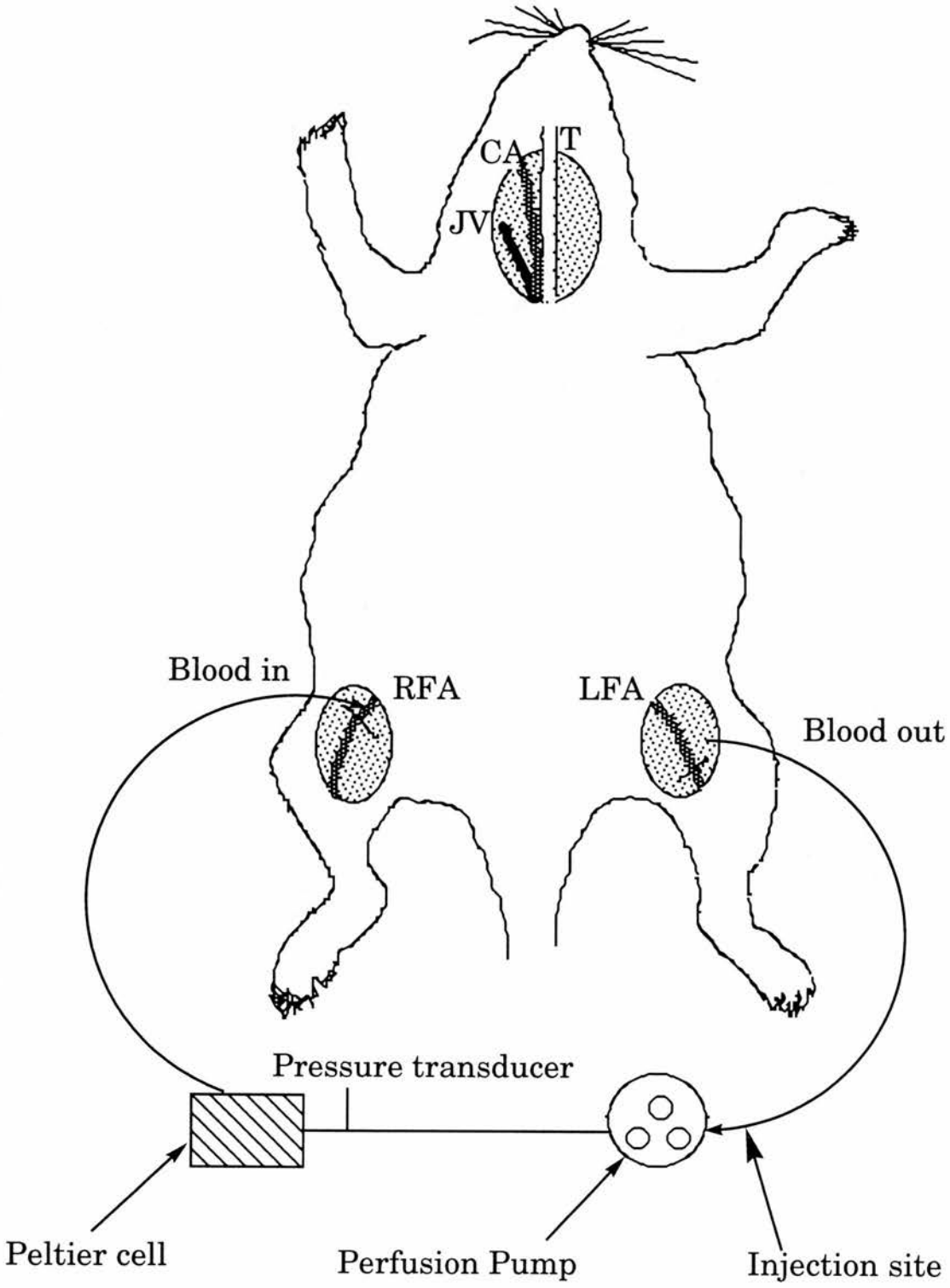



***Figure 2.5.*** *Diagram of the rat autoperfused hindlimb preparation showing cannulation of: trachea (T) for airflow measurement; carotid artery (CA) for measurement of mean arterial pressure; jugular vein (JV) for intravenous administration of drugs; left femoral artery (LFA) where blood enters perfusion circuit; and right femoral artery (RFA) where blood enters the hindlimb under observation. The relative positions of the components of the perfusion circuit are also shown.*

### 2.2.3. Experimental protocol for an investigation of vasoconstrictor substances

In order to validate the autoperfused hindlimb model, an attempt was made to reproduce the results of vascular studies quoted in the literature. It has been demonstrated that cooling augments noradrenaline-induced contraction in canine veins (Janssens & Vanhoutte, 1978) and that this is mediated by an apparent increase in responsiveness of $\alpha_2$-adrenoceptors (Flavahan *et al.*, 1985; Flavahan & Vanhoutte, 1986). Experiments were carried out using several $\alpha$-adrenoceptor antagonists (Table 2.6) in an effort to attenuate the cold-induced rise in perfusion pressure. In addition, the role of endothelin was investigated using the ET receptor antagonists, bosentan ($ET_{A/B}$) and BQ-123 ($ET_A$) (Table 2.6). All the antagonists were administered intra-arterially (i.a.) into the hindlimb circulation.

| Drug | Action | Dose |
|---|---|---|
| Phentolamine | $\alpha_1/\alpha_2$-adrenoceptor antagonist | 10 μg/kg |
| Prazosin | $\alpha_1$-adrenoceptor antagonist | 100 μg/kg |
| Yohimbine | $\alpha_2$-adrenoceptor antagonist | 300 μg/kg |
| Prazosin & yohimbine | $\alpha_1/\alpha_2$-adrenoceptor antagonists (combined) | as above |
| Bosentan | $ET_{A/B}$ receptor antagonist | 1000 μg/kg |
| BQ-123 | $ET_A$-receptor antagonist | 1000 μg/kg |

***Table 2.6.*** *Antagonists and inhibitors used in the autoperfused rat hindlimb preparation. Doses used were shown to be effective against the appropriate agonists (see Chapter 3).*

To prevent pressure changes caused by injection, drugs were administered so that they passed through the peristaltic pump before reaching the hindlimb. At the start of every

experiment 0.1ml of saline was injected intra-arterially to establish whether the vehicle itself had any direct effects. Because drug/saline solutions are of lower viscosity than blood, they produced a sharp fall in resistance and hence perfusion pressure when injected. To minimise this effect and to allow greater mixing with the blood, drugs were injected slowly (over 5-6 seconds).

### 2.2.4. Statistical Analysis

The results are expressed as the slope of temperature-HLPP curves which were constructed by plotting the natural logarithm of HLPP (ln HLPP) versus the inverse of temperature (1/T), according to Arrhenius analysis (a straight line is expected from the original Arrhenius equation, which is used as a means of expressing the temperature dependence of a response: $k = Ae^{-\mu/RT}$, where k = rate constant; A = constant; *e* = natural logarithm; $\mu$ = Arrhenius energy; R = gas constant; and T = temperature). Values are shown as mean ± standard error of the mean (SEM), and n = number of animals studied. Student's paired *t* test was used to compare the curves before and after the addition of an antagonist or inhibitor. Statistical significance was accepted when $P < 0.05$.

## 2.4. Drugs and solutions

| Drug | Use | Stock solution | Supplier |
|---|---|---|---|
| Acetylchloline (ACh) | Endothelium-dependent vasodilator | $dH_2O$ | Sigma Chemical (Dorset, UK) |
| Bosentan | Non-selective endothelin ($ET_{A/B}$) receptor antagonist | $dH_2O$ | Gift from Hoffmann-La Roche (Basel, Switzerland) |
| BQ-123 | Selective endothelin ($ET_A$) receptor antagonist | $dH_2O$ | Gift from Glaxo Group Research Ltd. (Ware, UK) |
| Calcium chloride ($CaCl_2$) | Salt (PSS[1]) | - | Fisons (Loughborough, UK) |
| Clonidine | Selective $\alpha_2$-adrenoceptor agonist | $dH_2O$ | Sigma Chemical (Dorset, UK) |
| Endothelin-1 (ET-1) | Vasoconstrictor | 50% MeOH:50% $dH_2O$ | Novabiochem (Nottingham,UK) |
| Ethidium bromide | Nuclear dye | $dH_2O$ | Sigma Chemical (Dorset, UK) |
| Ethylenediamine tetra acetic acid (EDTA) | Anti-oxidant | - | Sigma Chemical (Dorset, UK) |
| Glucose | Salt (PSS[1]) | - | Fisons (Loughborough, UK) |
| Glutaraldehyde | Fixitive[3] | - | Fisons (Loughborough, UK) |
| Heparin | Anticoagulant | 0.9% saline | CP Pharmaceuticals Ltd. (Wrexham, UK) |
| Indomethacin (Indo) | Cyclooxygenase inhibitor | 5% $NaHCO_3$ sol. at 37°C | Sigma Chemical (Dorset, UK) |
| Magnesium sulphate ($MgSO_4$) | Salt (PSS[1]) | - | Fisons (Loughborough, UK) |

| Drug | Use | Stock solution | Supplier |
|---|---|---|---|
| $N^G$-nitro-L-arginine methyl ester (L-NAME) | Nitric oxide synthase (NOS) inhibitor | $dH_2O$ | Sigma Chemical (Dorset, UK) |
| Noradrenaline (NA) | Non-selective $\alpha_1/\alpha_2$-adrenoceptor agonist | $dH_2O$ | Sigma Chemical (Dorset, UK) |
| Paraformaldehyde | Fixitive[3] | - | Fisons (Loughborough, UK) |
| Phentolamine | Non-selective $\alpha_1/\alpha_2$-adrenoceptor antagonist | 50% EtOH:50% $dH_2O$ | Sigma Chemical (Dorset, UK) |
| Phenylephrine (PE) | Selective $\alpha_1$-adrenoceptor agonist | $dH_2O$ | Sigma Chemical (Dorset, UK) |
| Potassium chloride (KCl) | Salt (PSS) and vasoconstrictor[2] | - | Fisons (Loughborough, UK) |
| Potassium orthophosphate ($KH_2PO_4$) | Salt (PSS[1]) | - | Fisons (Loughborough, UK) |
| Prazosin | Selective $\alpha_1$-adrenoceptor antagonist | 50% EtOH:50% $dH_2O$ | Pfizer Ltd. (Kent, UK) |
| Sodium bicarbonate ($NaHCO_3$) | Salt (PSS[1]) | - | Fisons (Loughborough, UK) |
| Sodium chloride (NaCl) | Salt (PSS[1]) | - | Fisons (Loughborough, UK) |
| Sodium nitroprusside (SNP) | Endothelium-independent vasodilator | $dH_2O$ | Sigma Chemical (Dorset, UK) |
| Sodium pentobarbitone | General anaesthetic | - | Rhone Merieux Ltd. (Essex, UK) |

| Drug | Use | Stock solution | Supplier |
|---|---|---|---|
| Sodium phosphate dibasic ($Na_2HPO_4.2H_2O$) | Salt (sodium phosphate buffer[4]) | - | Fisons (Loughborough, UK) |
| Sodium phosphate monobasic ($NaH_2PO_4.2H_2O$) | Salt (sodium phosphate buffer[3]) | - | Fisons (Loughborough, UK) |
| Yohimbine | Selective $\alpha_2$-adrenoceptor antagonist | 50% EtOH:50% $dH_2O$ | Sigma Chemical (Dorset, UK) |

***Table 2.8.*** *List of drugs and chemicals used in this thesis. $dH_2O$ = distilled water; EtOH = ethanol; MeOH = methanol; PSS = physiological salt solution[1]. Note: subsequent dilutions of the peptides endothelin-1 and BQ-123 were made in PSS containing 0.1% bovine serum albumin (Sigma Chemical, Dorset, UK) in order to minimise non-specific binding to the glassware, tubing etc..*

**1.** Physiological salt solution (PSS): 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 $KH_2PO_4$, 25.0 $NaHCO_3$, 0.026 EDTA, and 5.5 glucose (all in mM).

**2.** Depolarising potassium chloride solution: PSS with an equimolar replacement of NaCl with KCl, resulting in a final potassium concentration of 60mM.

**3.** Sodium phosphate buffer (0.2M):

| | |
|---|---|
| Solution A | 32.2g sodium phosphate monobasic ($NaH_2PO_4.2H_2O$) in 1000ml $dH_2O$ |
| Solution B | 35.6g sodium phosphate dibasic ($Na_2HPO_4.2H_2O$) in 1000ml $dH_2O$ |

The desired pH was obtained by mixing the two solutions in the ratio given below:

| Solution A (ml) | Solution B (ml) | pH of buffer |
|---|---|---|
| 72 | 28 | 7.2 |



**4.** Karnovsky's fixitive (to make 1l):

| | |
|---|---|
| 500ml | 0.2M sodium phosphate buffer[3] |
| 200ml | 10% paraformaldehyde (100g in 1000ml $dH_2O$ at 60°C - drops of 10N NaOH added until solution cleared) |
| 50ml | 50% glutaraldehyde |

The pH was adjusted to 7.2 by adding 10M HCl or 10M NaOH. Total volume was made up to 1000ml with $dH_2O$.

# CHAPTER 3:
# VALIDATION OF TECHNIQUES USED

## 3.1. Small vessel arteriograph (perfusion myograph)

### 3.1.1. Reproducibilty of concentration-response curves

It was originally anticipated that the effect of cooling on the response to ET-1 would be examined by generating concentration-response curves to ET-1 firstly at 37°C, and then repeating in the same vessel after cooling to 24°C; the order of the temperatures would be randomised between vessels. In order to determine whether it was feasible to carry out more than one concentration-response curve to ET-1 in a single vessel, up to four consecutive curves were generated in individual vessels, allowing each to recover to its original resting lumen diameter - or the maximum diameter attainable after a 2 hour recovery period (Table 3.1) - after the completion of each curve. It should be noted that the maximum concentration of ET-1 attainable with the batch of ET-1 used in this set of experiments was not sufficient to achieve maximal contraction to ET-1. Subsequent experiments used a single batch of ET-1 that enabled a 10-fold increase in concentration of the stock solution.

***Table 3.1.*** *Resting lumen diameter of vessels before each concentration-response curve to ET-1 at 37°C, and the time taken to recover between curves*

| Curve | 1st | 2nd | 3rd | 4th |
|---|---|---|---|---|
| % Resting LD | 100 ± 0 | 100 ± 1 | 97 ± 2 | 101 ± 3 |
| Recovery time | - | 93 ± 7 | 91 ± 10 | 75 ± 11 |

*Lumen diameter (LD); recovery time in minutes. All values are mean ± SEM. n = 9-12 for 1st, 2nd and 3rd curves; n = 4 for 4th curve.*

From Figure 3.1, it can be seen that there is a significant rightward-shift of second, and subsequent concentration-response curves to ET-1 compared to the first curve ($EC_{50}$ values have not been calculated because the responses to ET-1 did not reach a maximum). Since the responsiveness to ET-1 was found to be significantly depressed after the first concentration-response curve, it was decided to examine the effect of cooling on the response to ET-1 by generating single concentration-response curves in individual vessels, either at 37°C or 24°C.

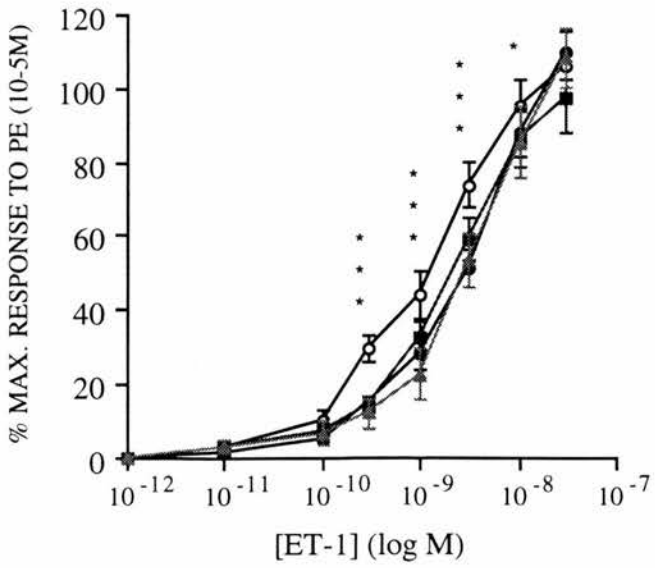



***Figure 3.1.*** *The reproducibility of responses to ET-1 in rat mesenteric resistance arteries: consecutive concentration-response curves to ET-1 (expressed as % response to PE, $10^{-5}$M) at 37°C: 1st curve (○; n=12); 2nd curve (●; n=12); 3rd curve (■; n=9); and 4th curve (▲; n=4). All values are mean ± SEM. *P < 0.05; ***P < 0.001 (paired t-test; 1st vs. subsequent curves). Note: in this set of experiments the maximal response to ET-1 was not attainable with the concentrations available (see text).*

### 3.1.2. De-endothelialisation techniques

After attempts were made to de-endothelialise vessels using the air bubble technique (see Chapter 2; under section 2.1.5.1.), the integrity of the endothelium was assessed in four ways. The results from each of these assessments are given below.

#### 3.1.2.1. Functional tests

The endothelium-dependent dilator ACh ($10^{-6}$M) was given during contraction to the $\alpha_1$-adrenoceptor agonist, PE ($10^{-5}$M), in order to determine endothelial integrity functionally. In vessels with an intact endothelium, ACh induced relaxation to, or close to, the original resting lumen diameter of the vessel (Table 3.2). After the passage of air through the vessel lumen, ACh was unable to induce relaxation, and indeed quite often elicited a small contraction (Table 3.2). These effects were independent of temperature.

***Table 3.2.*** *The relaxant response to ACh before and after passage of air through the vessel lumen*

| | Before | After |
|---|---|---|
| 37°C | 88 ± 4 | -3 ± 1 *** |
| 24°C | 92 ± 5 | -2 ± 0 *** |

*% Relaxation to acetylcholine, ACh ($10^{-6}$M). Values are mean ±SEM. n = 6 vessels at each temperature. ***P < 0.001 (paired t-test).*

The results from these tests implied that the air bubble destroyed the endothelium; morphological techniques were employed to investigate whether this was due to removal of the endothelium or to its damage (see below).

### 3.1.2.2. Confocal microscopy

Ethidium bromide labels the nuclei of cells which have a permeabilised membrane, i.e. those which are damaged. Figure 3.2 shows an artery with an intact endothelium, which had been stained with ethidium bromide (5μg/ml) for 30 minutes after removal from the myograph and then imaged through a confocal microscope. Only adventitial cells from the top (a) and bottom (c) of the vessel were stained; these cells were likely to have been damaged during the dissection procedure. No endothelial cells were seen when the plane of focus was set away from the adventitia to the luminal surface (b), indicating the presence of an intact endothelium. Figure 3.3 shows confocal images of two vessels which had air infused through the lumen, before removing from the myograph and immersing in ethidium bromide. The permeabilised endothelial cells had lost their usual ordered orientation, and had formed clusters. Thus, it appears that the passage of an air bubble through the vessel lumen damages the endothelial cells, but does not completely strip them from the vessel wall. The absence of staining of smooth muscle cells suggests this method of de-endothelialisation does not have adverse effects on the underlying smooth muscle.

***Figure 3.2.*** *Confocal images of an endothelium-intact rat mesenteric resistance artery, stained with ethidium bromide after removal from the myograph. Only adventitial cells from the top (a) and bottom (c) of the vessel are stained. When the plane of focus is set away from the adventitia to the luminal surface (b), no endothelial cells are seen, indicating the presence of an intact endothelium. Scale bar= 40μm.*

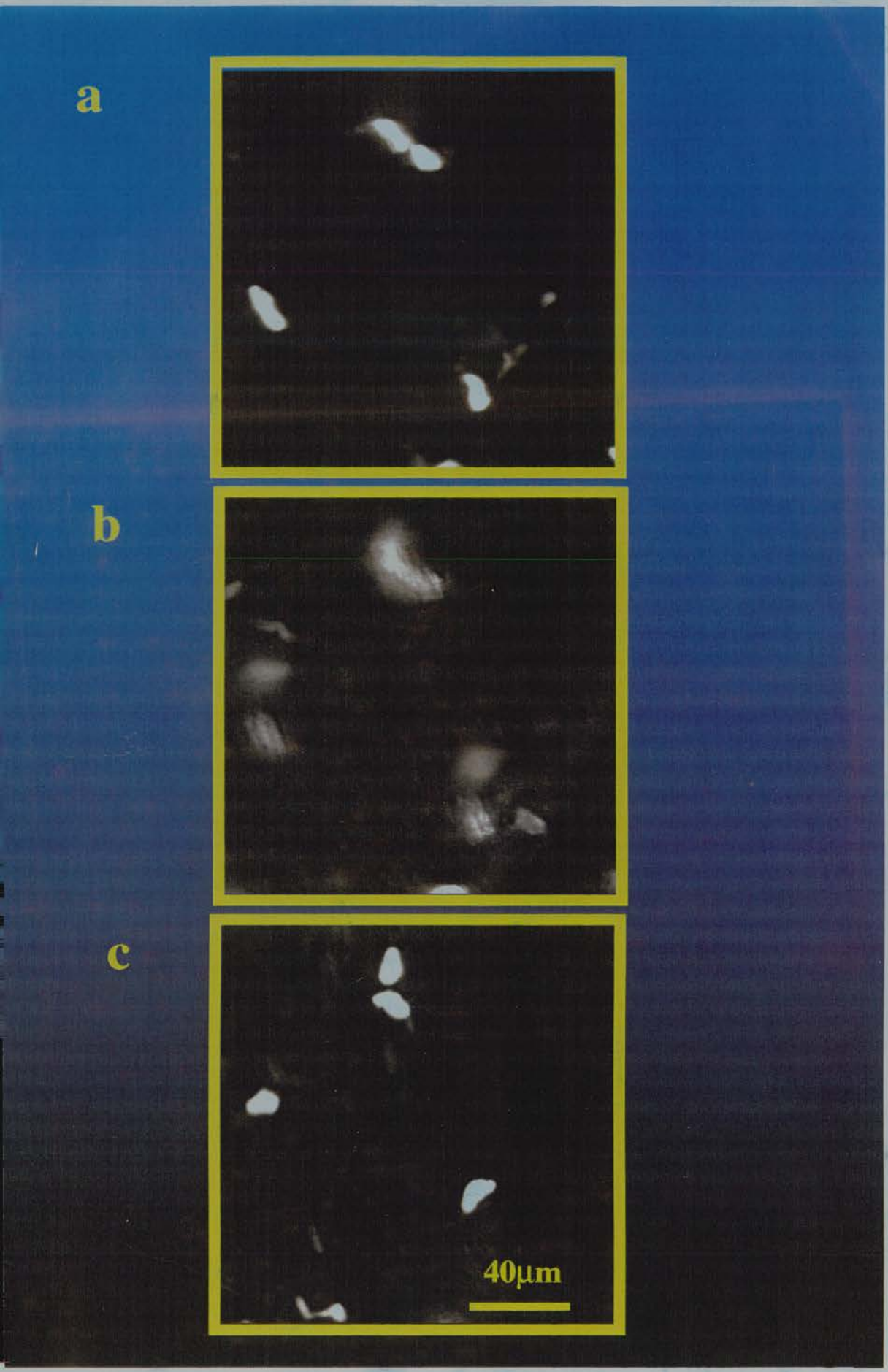
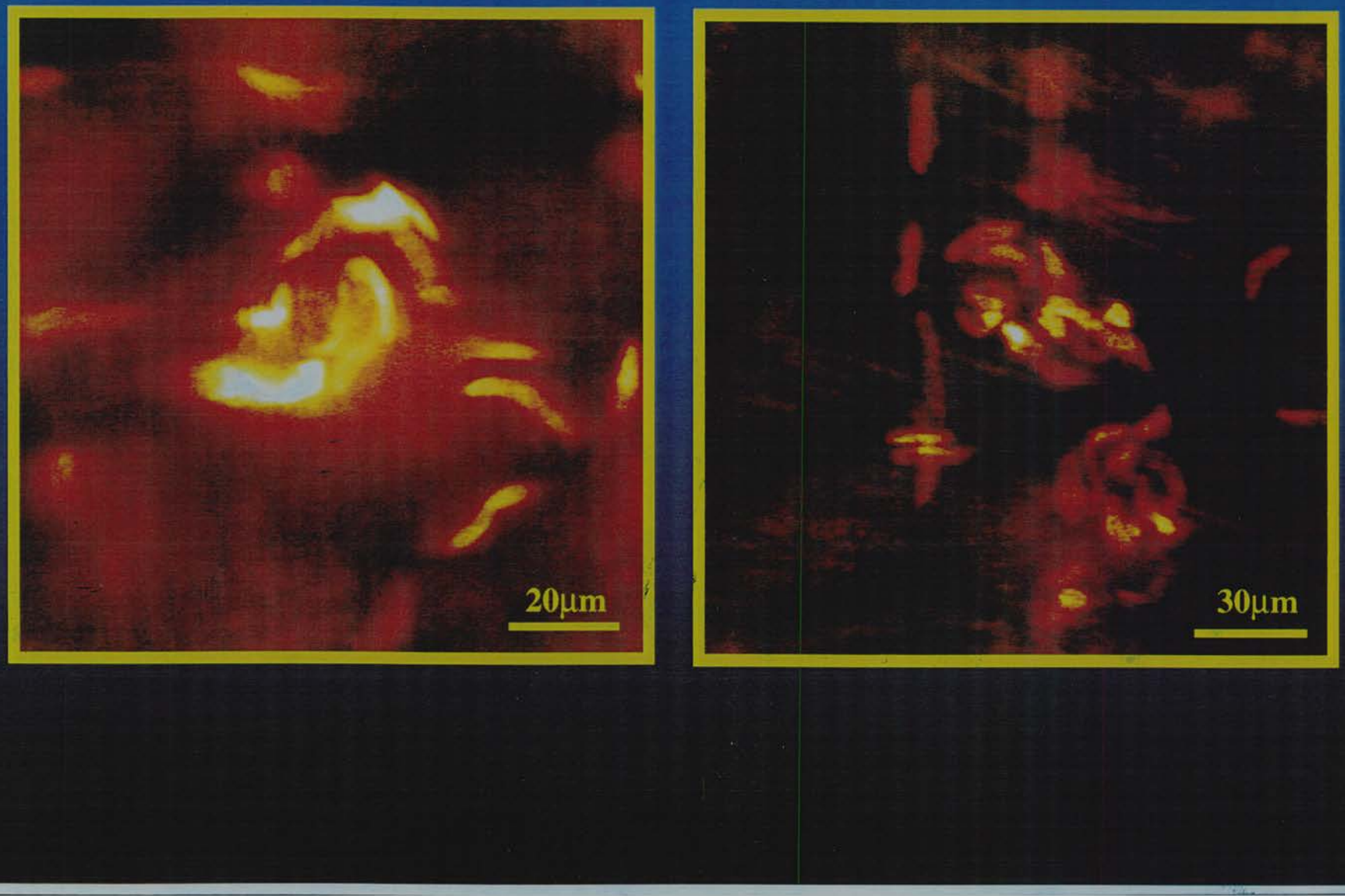

a
b
c
40μm

***Figure 3.3.*** *Confocal images showing the effects of the passage of air through two rat mesenteric resistance arteries, stained with ethidium bromide after removal from the myograph. Both the left (x40) and right (x25) panel show stained endothelial cells which have lost their ordered structure and formed clusters. Scale bar = 20μm (left panel) and 30μm (right panel).*


20μm
30μm

### 3.1.2.3. Scanning electron microscopy

Examination of the luminal surface of arteries by scanning electron microscopy (SEM) revealed the presence of an intact endothelial cell layer in all control arteries, which was effectively removed following the passage of air through the lumen (Figure 3.4). Substantial endothelial cell remnants were attached to the luminal surface in a clustered formation (Figure 3.5), similar to those observed by confocal microscopy.

### 3.1.2.4. Transmission electron microscopy

In the intact control arteries which had been fixed after removal from the myograph, transmission electron microscopy (TEM) showed the intima, consisting of a single layer of endothelial cells and the inner elastic lamina, and the media, made up of smooth muscle cells (Figure 3.6). Large vacuoles were seen under some of the endothelial cells in these control arteries (Figure 3.6a). Because they were also present in arteries which had been fixed immediately after dissection from the mesentery (Figure 3.6b), vacuoles would appear to be artefacts of the fixation procedure, rather than a result of the pressure myograph technique; indeed, others have also found similar vacuoles in non-pressurised arteries (Greensmith *et al.*, 1984). The number of vacuoles has been found to increase after storage of vessels for several hours in a refrigerator (Abebe *et al.*, 1993; K. Cracknell, personal communication). A large number of the experiments carried out in this thesis were done in vessels which had been stored at 4°C for similar time periods. Relaxation to ACh was not impaired in these arteries, however, suggesting the functional response can remain normal despite the occurence of some morphological changes.

From Figure 3.7 it can be seen that the passage of air through the vessel lumen effectively removed the endothelium from the inner elastic lamina, but that substantial endothelial debris remained in the lumen, which is similar to the findings from SEM and confocal microscopy. In several micrographs the underlying smooth muscle cells showed some signs of damage: the presence of vacuoles (Figure 3.7b) or swollen

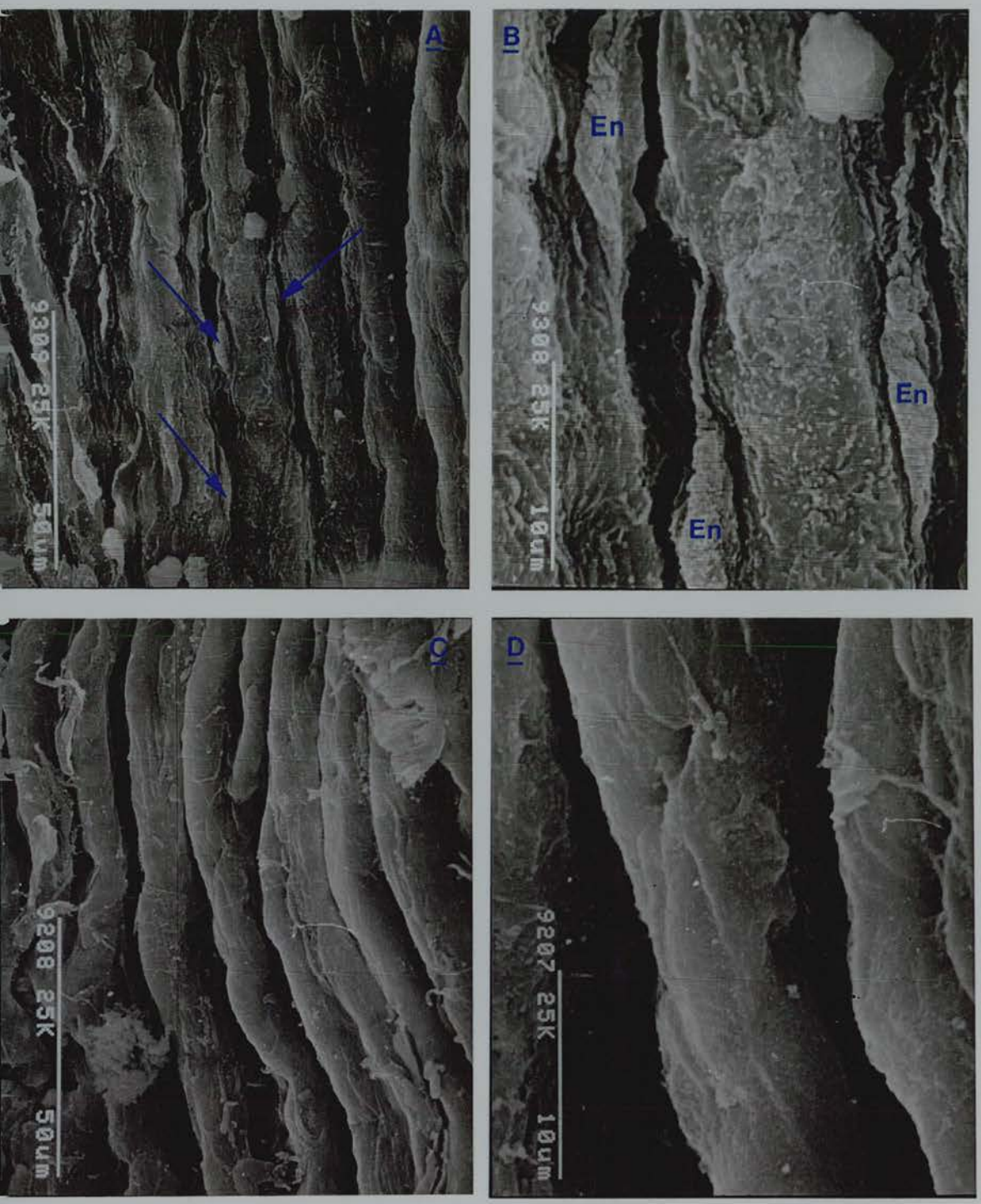



***Figure 3.4.*** *Scanning electron micrographs of the luminal surface of rat mesenteric resistance arteries, fixed by immersion after removal from the myograph.* ***A.*** *In endothelium-intact vessels the folded luminal surface is covered by a continuous layer of endothelial cells (arrows). Scale bar = 50µm.* ***B.*** *Several endothelial cells (En) under higher magnification. Scale bar = 10µm.* ***C.*** *In arteries which had an air bubble passed through the lumen there are no intact endothelial cells remaining. Scale bar = 50µm.* ***D.*** *Higher magnification of a de-endothelialised vessel reveals the absence of any endothelial cells in the folds of the luminal surface. Scale bar = 10µm.*


***Figure 3.5.*** *Scanning electron micrograph showing the effect of the passage of air through a rat mesenteric resistance artery, fixed after removal from the myograph. Remnants of endothelial cells (En) can be seen adhering to the luminal surface of the vessel. Scale bar = 50μm.*

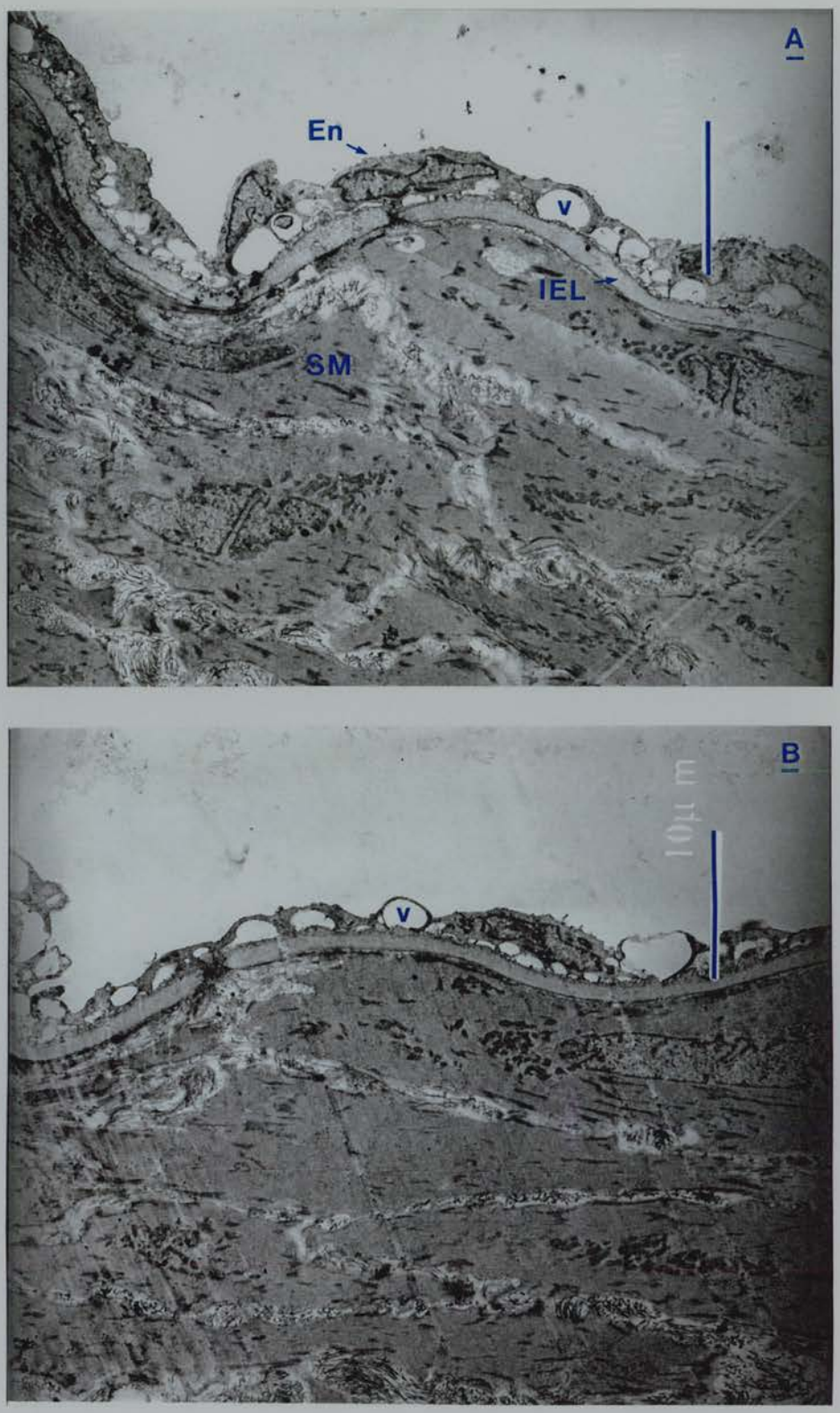



***Figure 3.6.*** *Transmission electron micrographs of endothelium-intact rat mesenteric resistance arteries, fixed by immersion after removal from the myograph (A) and fixed immediately after removal from the animal (B).* ***A.*** *This micrograph shows an intact layer of endothelial cells (En) beneath which are the inner elastic lamina (IEL) and smooth muscle cells (SM). In some areas vacuoles (V) have formed in the endothelial cells and in the subendothelial space. Scale bar= 10μm.* ***B.*** *Similar vacuoles (V) are also present in vessels which had not been mounted on the myograph. Scale bar = 10μm.*

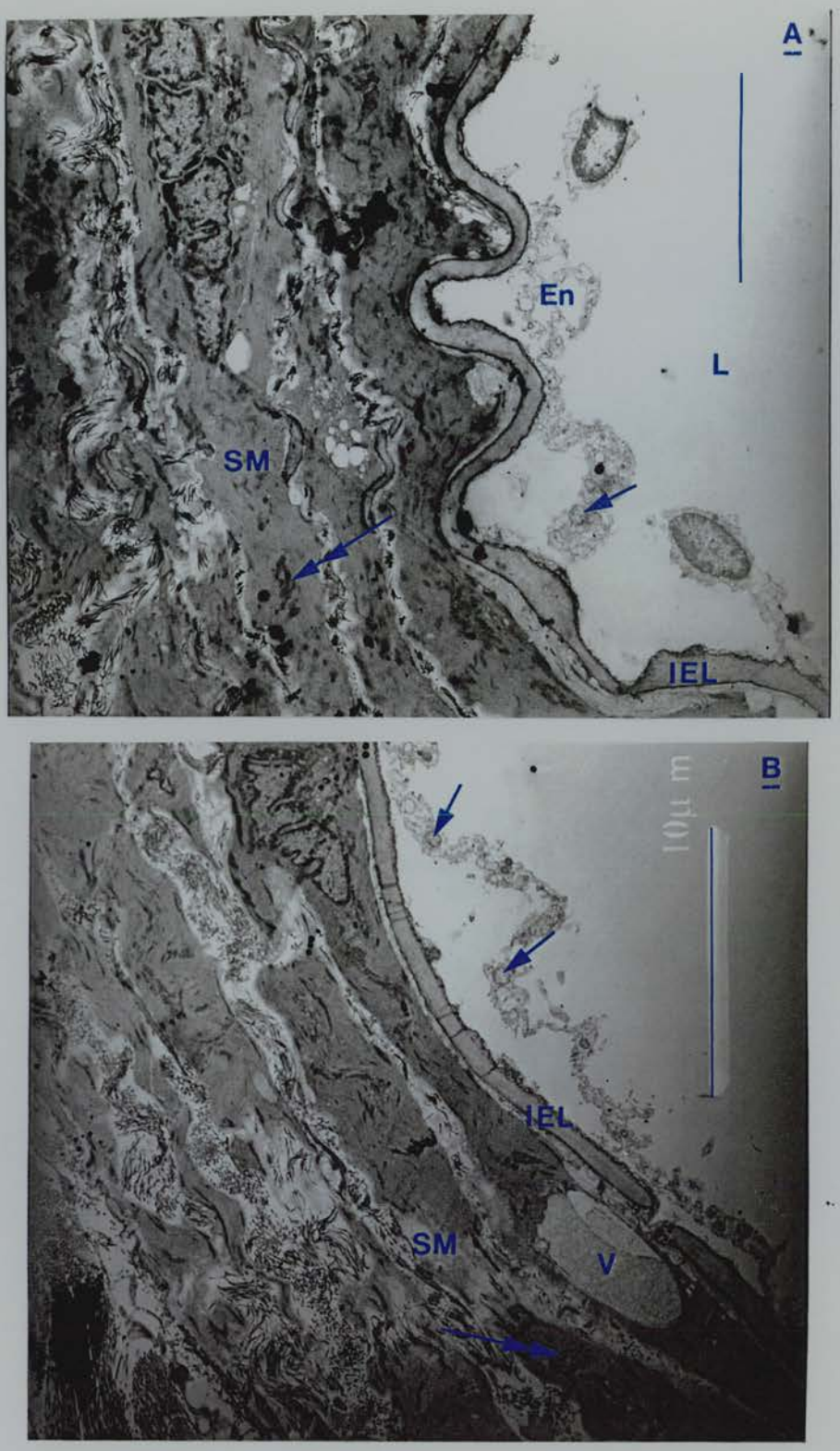



***Figure 3.7.*** *Transmission electron micrographs of rat mesenteric resistance arteries which were perfused with air, and fixed by immersion after removal from the myograph.* ***A.*** *This micrograph shows remnants of endothelial cells (En) in the lumen (L), above an intact inner elastic lamina (IEL). Swollen mitochondria are present in the endothelial cell remnants (single arrows). The mitochondria (double arrow) in the underlying smooth muscle cells (SM) appear normal. Scale bar = 10μm.* ***B.*** *A large vacuole (V) can be seen in the smooth muscle layer (SM), directly beneath a disrupted section of the inner elastic lamina (IEL). Scale bar= 10μm.*

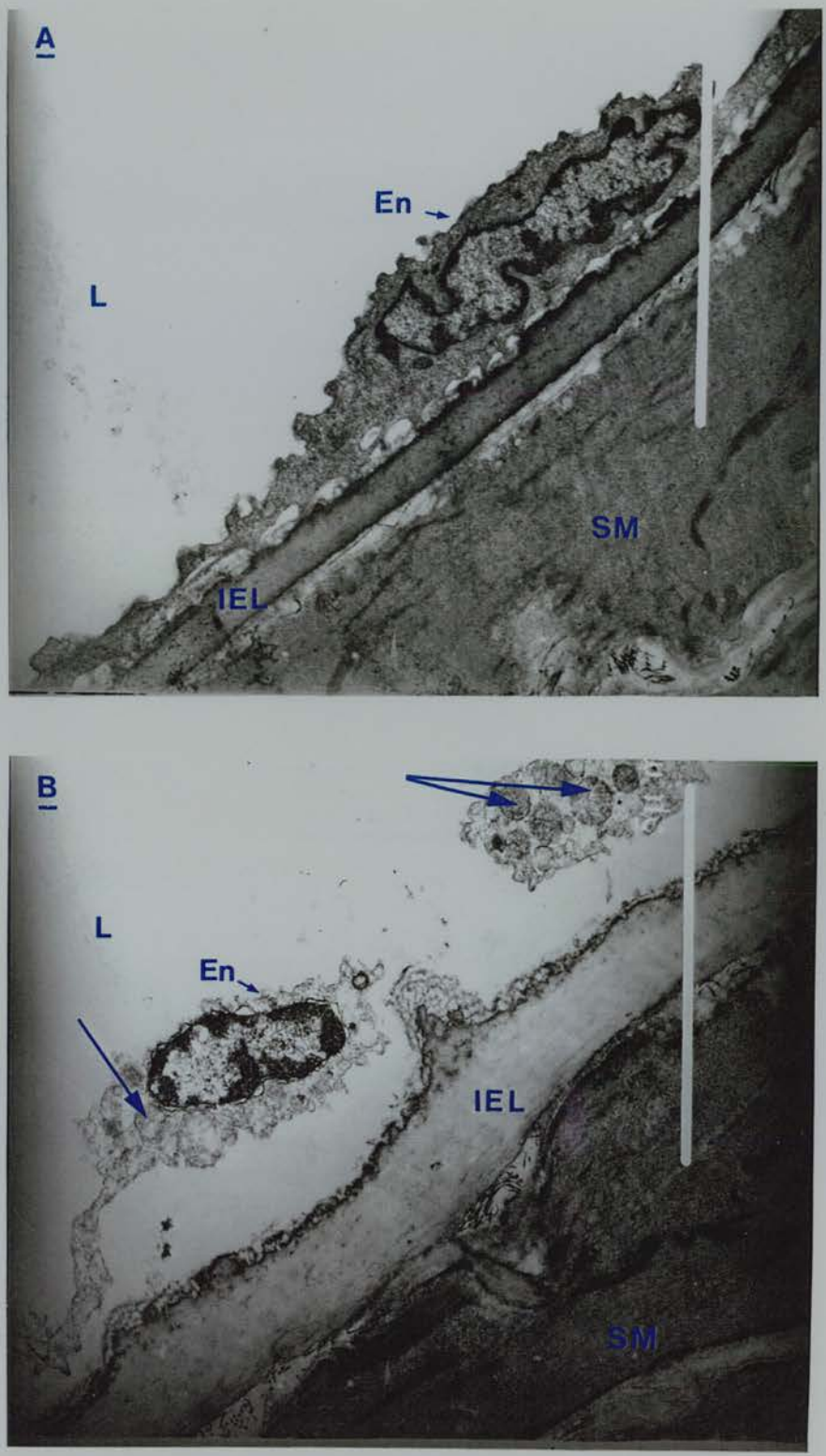



*Figure 3.8. High magnification transmission electron micrographs of rat mesenteric resistance arteries fixed by immersion after removal from the myograph. Endothelial cells (En) in intact arteries (**A**) appear normal, in contrast to those found in vessels through which an air bubble was passed (**B**), where swollen mitochondria are present (arrows). Scale bars = 5μm. Lumen (L); inner elastic lamina (IEL); smooth muscle (SM).*

mitochondria. This damage did not, however, affect functional responses to PE which were tested in these same vessels before fixation, and in all vessels of subsequent studies (see below). Rubbing the vessel lumen with a human hair appears to be more effective in completely removing the endothelium than the passage of an air bubble, but the hair technique results in more damage to the smooth muscle (K. Cracknell, personal communication). TEM under higher magnification revealed the presence of swollen mitochondria in the detatched endothelial cells of denuded arteries, which were not observed in intact control vessels (Figure 3.8), indicating that the endothelial cells which remained were damaged, and were therefore unlikely to be functional (as was evidenced by the abolition of the relaxant response to ACh).

The air-bubble technique thus appears to be an effective way of denuding resistance arteries, whilst causing less damage to the underlying smooth muscle than other mechanical methods. Perhaps increasing the time of PSS perfusion through the vessel lumen, from 2-3 min to a longer period, after the passage of air would further assist the removal of the endothelial debris.

### 3.1.3. Effects of de-endothelialisation and cooling on artery diameter

Removal of the endothelium and/or cooling did not cause a significant change in resting lumen diameter (Tables 3.3 and 3.4), or to the contractile response to the 'wake-up' dose of PE ($10^{-5}$M) (Tables 3.5 and 3.6). This was obviously an advantage, as it meant the results could be expressed as a % of the maximal response to the 'wake-up' dose of PE regardless of the temperature or endothelial state of the vessel. % Contraction to PE was calculated as:

$$\frac{LD_1 - LD_2}{LD_1} \times \frac{100}{1}$$

where $LD_1$ = resting lumen diameter; $LD_2$ = lumen diameter at maximal contraction.

***Table 3.3.*** *The effect of de-endothelialisation on vessel diameter at 37°C and 24°C*

| | Before | After |
|---|---|---|
| 37°C | 278 ± 21 | 277 ± 21 |
| 24°C | 283 ± 14 | 279 ± 16 |

*Vessel diameter in μm. Values are mean ± SEM. n = 6 vessels at each temperature.*

***Table 3.4.*** *The effect of cooling on diameter of intact vessels*

| Vessel diameter at 37°C | Vessel diameter after cooling to 24°C |
|---|---|
| 278 ± 20 | 276 ± 22 |

*Vessel diameter in μm. Values are mean ± SEM. n = 12 vessels at each temperature.*

***Table 3.5.*** *The effect of de-endothelialisation on the contractile response to PE at 37°C and 24°C*

| | Before | After |
|---|---|---|
| 37°C | 74 ± 1 | 75 ± 1 |
| 24°C | 75 ± 1 | 75 ± 1 |

*% Contraction to phenylephrine, PE ($10^{-5}$M) . Values are mean ± SEM. n = 6 vessels at each temperature.*

***Table 3.6.*** *The effect of cooling on the contractile response to PE ($10^{-5}$M)*

| % Contraction at 37°C | % Contraction after cooling to 24°C |
|---|---|
| 73 ± 1 | 73 ± 1 |

*% Contraction to phenylephrine, PE ($10^{-5}$M). Values are mean ± SEM. n = 12 vessels at each temperature.*

### 3.1.4. Justification for using arteries obtained from gluteal fat biopsies

In order to study the pathophysiological role of the vascular endothelium in Raynaud's disease, resistance arteries were obtained from fat biopsies taken from the gluteal region of Raynaud's patients and healthy control subjects (see Methods 2.1.2.3). Ideally, one would examine vessels isolated from the digits, i.e. the actual region predominantly affected by the condition, but this approach would almost certainly be unacceptable to volunteers. Digital biopsies could be obtained from post-mortem tissue, but such a source raises potential problems that would need to be addressed: cause of death; length of time taken to receive tissue after death; accumulation of toxic substances; and the temperature at which the body was stored. Variability of these factors would affect the viability of arteries dissected, and ultimately the responses of any arteries studied. Although it may be interesting to study such samples, they inevitably could not be used to conduct an age- and sex-matched, controlled study between Raynaud's patients and control subjects.

It is likely that Raynaud's disease manifests itself in, for example, the fingers, toes, ears, lips and nose, because these parts are extemities which are exposed to the cold. The gluteal region is usually well protected from the elements and is, therefore, an area which one would not normally expect to find cold-induced vasospasm. Nevertheless, when interviewing the Raynaud's patients recruited for the studies in this thesis, it was learned that several of them did indeed suffer from cold buttocks as a result of poor circulation to this region. Perhaps this is not so surprising since there is a higher incidence of Raynaud's disease in patients with migraine and/or variant angina, suggesting that it may be part of a *generalised* vascular defect. If this is true, one would expect the abnormality to be evident in vessels not generally associated with the symptoms of Raynaud's disease. For this reason, it would seem that subcutaneous resistance arteries isolated from gluteal biopsies are a suitable alternative to digital vessels. In addition, the buttocks have a large proportion of subcutaneous fat, and the scar resulting from the biopsy procedure is at an unobtrusive site.

## 3.2. Autoperfused hindlimb of the anaesthetised rat

### 3.2.1. Reproducibility of cold-induced vasoconstriction

A series of experiments were performed in order to determine the reproducibility of the temperature-hindlimb perfusion pressure (HLPP) curves; four consecutive curves were generated in each animal, as described in Chapter 2. From Figure 3.9, it can be seen that the temperature-HLPP curves were reproducible, when generated both by direct cooling from 37°C to 21°C in 4C° steps (Figure 3.9a), and by random changes to each temperature (Figure 3.9b). There was no significant difference between the slope of the curves (Table 3.7).

When examining the effect of drugs on the cold-induced rise in HLPP, two control temperature-HLPP curves were generated prior to drug administration into the hindlimb. Thus, the effect of a drug was examined by comparing the second and third temperature-HLPP curves; because there was no significant difference between these curves in the control experiments (Figure 3.10), this was a satisfactory way to study the effect of drugs on the increase in HLPP during cooling.

### 3.2.2. Influence of HLPP on systemic blood pressure

The cold-induced rise in HLPP had no effect on mean arterial blood pressure (MAP); a representative trace of HLPP and MAP is shown in Figure 3.11. The flow rate of blood through the hindlimb was chosen to achieve a HLPP approximately equal to that of MAP. The flow rate required for this was 2.5 ± 0.1 ml/min. One study has reported hindlimb blood flow in rats to be 3.8 ± 0.2 ml/min (Drexler & Lu, 1992), which suggests the flow used in the present model was low. This might have resulted in hypoxaemia, inducing the release of substances such as ET and NO, and is something that should be considered when evaluating the data obtained from this model.

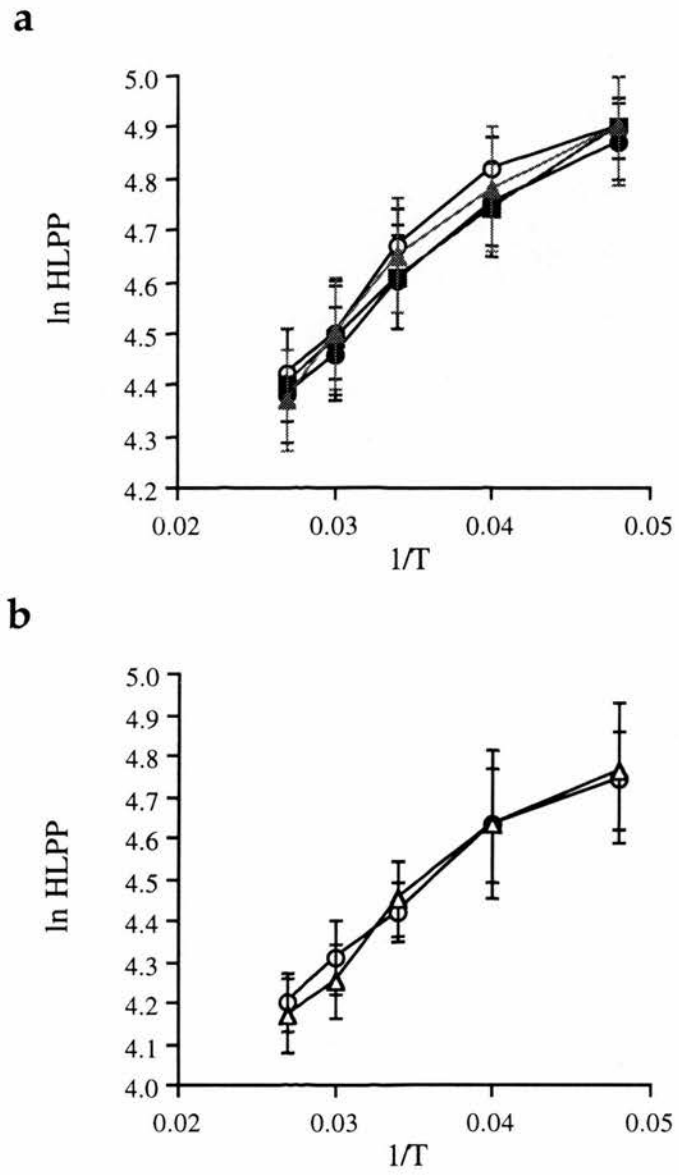



***Figure 3.9.*** *The effect of cooling the blood on hindlimb perfusion pressure (HLPP) in anaesthetised rats: consecutive temperature (T)-HLPP curves from 37°C to 21°C, directly (Figure 3.9a): 1st (○; n=6), 2nd (●; n=6), 3rd (■; n=6), and 4th curve (▲; n=6); or at random (Figure 3.9b): 1st (○; n=3) and 2nd curve (Δ; n=3). All values are mean ± SEM.*

***Table 3.7.*** *Slope of consecutive temperature-HLPP curves*

| Curve | 1st | 2nd | 3rd | 4th | Random 1 | Random 2 |
|---|---|---|---|---|---|---|
| Slope | 23.69±3.24 | 23.67±3.25 | 23.83±4.38 | 24.42±3.70 | 26.11±3.25 | 28.82±5.05 |

*Slope refers to simple line of best fit through each curve. All values are mean ± SEM.*

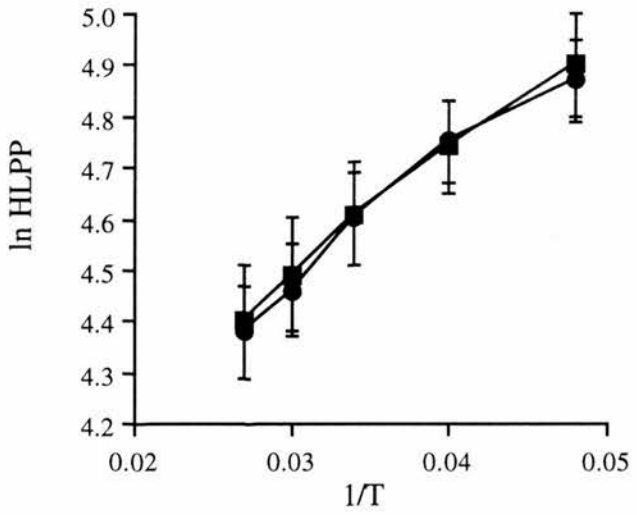



***Figure 3.10.*** *The effect of cooling the blood on hindlimb perfusion pressure (HLPP) in anaesthetised rats: 2nd (●; n=6) and 3rd (■; n=6) consecutive temperature-HLPP curves from 37°C to 21°C, directly. All values are mean ± SEM.*

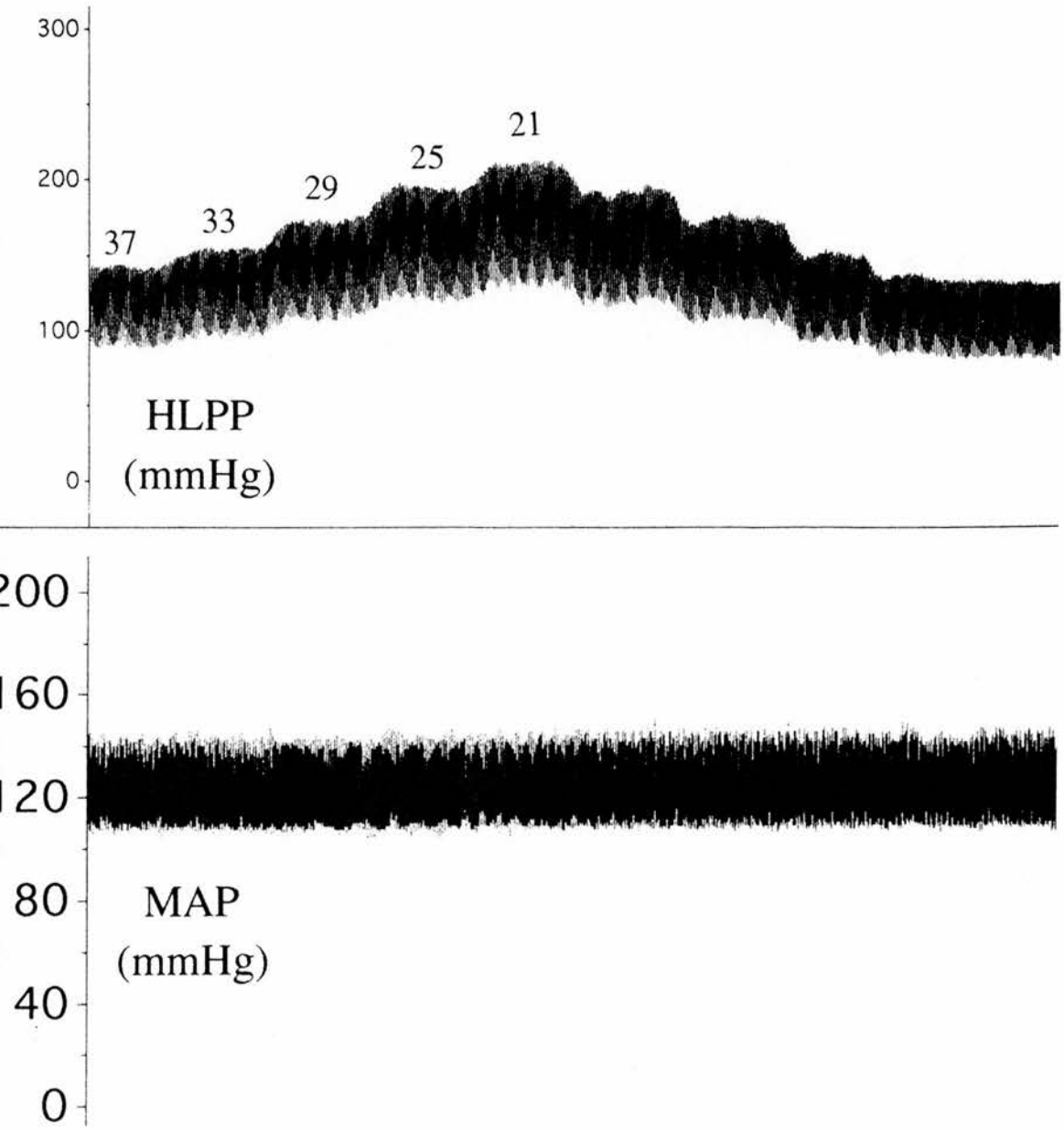



***Figure 3.11.*** *Representative tracing showing the effect of cooling from 37°C to 21°C in 4C° steps on hindlimb perfusion pressure (HLPP) and mean arterial pressure (MAP) (mmHg).*

The doses of antagonist drugs injected i.a. into the hindlimb circulation were shown to be effective in significantly attenuating the pressor response to the appropriate agonists administered intra-arterially (i.a.) into the hindlimb, whilst having non-significant effects on the pressor response to agonists injected intravenously (i.v.) into the systemic circulation (Figures 3.12 - 3.14). This implies the doses used were small enough to have greater local, over systemic, actions.

The effect of the ET receptor antagonists on the systemic circulation was not investigated because ET-1 elicited a prolonged pressor response in the hindlimb, and a similar response systemically would have led to pressure changes in the hindlimb circulation which may have affected the degree of responsiveness shown to the antagonists. The effectiveness of the $ET_A$ receptor antagonist BQ-123 (1000μg/kg i.a.), and the mixed $ET_{A/B}$ receptor antagonist bosentan (1000μg/kg i.a.), in blocking the pressor response to ET-1 (100ng/kg i.a.) in the hindlimb are shown in Figures 3.15 and 3.16. The degree of attenuation was not significant for either drug ($P$=0.10 and $P$=0.07 for BQ-123 and bosentan respectively) because of the low number of experiments carried out (n=2 for each drug owing to the expense and lack of availability of these antagonists).

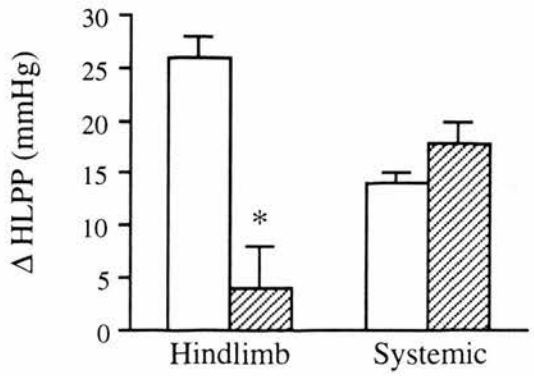



***Figure 3.12.*** *Local vs. systemic antagonist action of phentolamine on the pressor response to noradrenaline: the change in hindlimb perfusion pressure (Δ HLPP) (mmHg) resulting from hindlimb (i.a.) or systemic (i.v.) administration of noradrenaline (100ng), before (unhatched columns) and after (hatched columns) hindlimb (i.a.) administration of phentolamine (10μg/kg). All values are mean ± SEM (n=4). *P < 0.05 (paired t-test).*

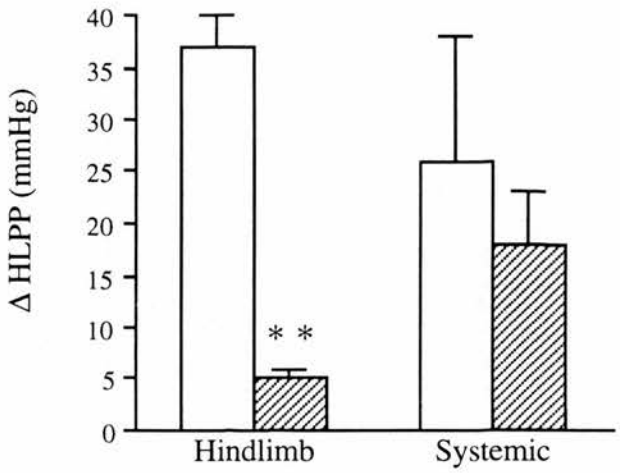



***Figure 3.13.*** *Local vs. systemic antagonist action of prazosin on the pressor response to noradrenaline: the change in hindlimb perfusion pressure (Δ HLPP) (mmHg) resulting from hindlimb (i.a.) or systemic (i.v.) administration of noradrenaline (100ng), before (unhatched columns) and after (hatched columns) hindlimb (i.a.) administration of prazosin (100μg/kg). All values are mean ± SEM (n=5). **P < 0.01 (paired t-test).*

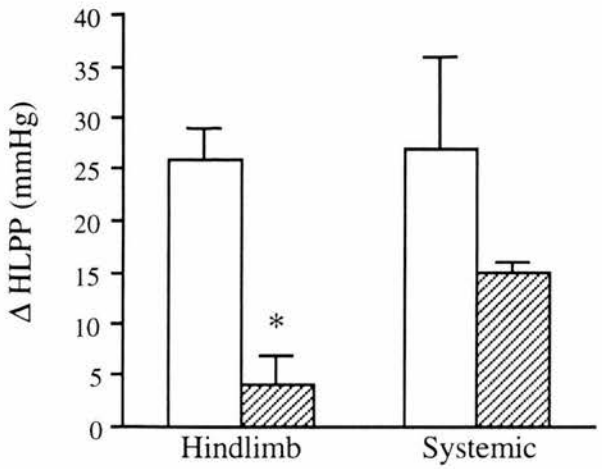



***Figure 3.14.*** *Local vs. systemic antagonist action of yohimbine on the pressor response to clonidine: the change in hindlimb perfusion pressure (Δ HLPP) (mmHg) resulting from hindlimb (i.a.) or systemic (i.v.) administration of clonidine (100ng), before (unhatched columns) and after (hatched columns) hindlimb (i.a.) administration of yohimbine (300μg/kg). All values are mean ± SEM (n=5). *P < 0.05 (paired t-test).*

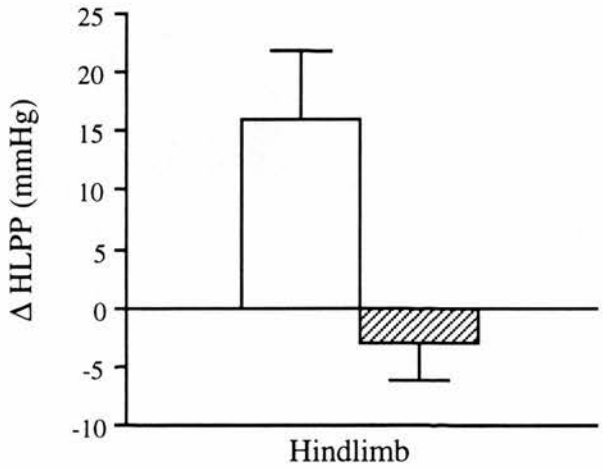



***Figure 3.15.*** *The antagonist action of BQ-123 on the pressor response to ET-1: hindlimb (i.a.) administration of ET-1 (100ng) alone (unhatched column) and after BQ-123 (1000μg/kg) (hatched column). All values are mean ± SEM (n=2).*

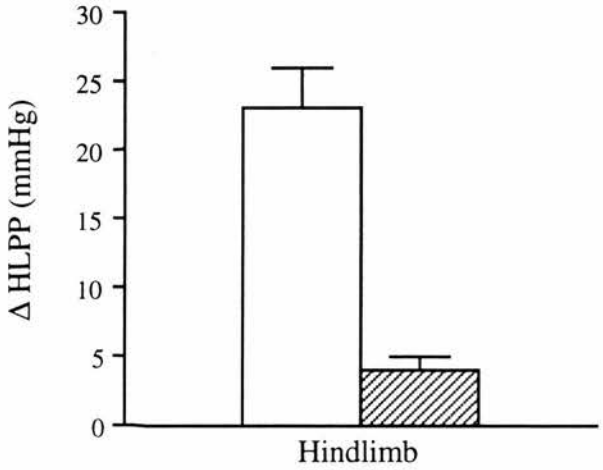



***Figure 3.16.*** *The antagonist action of bosentan on the pressor response to ET-1: hindlimb (i.a.) administration of ET-1 (100ng) alone (unhatched column) and after bosentan (1000μg/kg) (hatched column). All values are mean ± SEM (n=2).*

### 3.2.3. Contribution of blood viscosity to cold-induced increase in HLPP

Temperature-HLPP curves were generated during saline-perfusion of the hindlimb to establish whether the rise in HLPP induced by cooling was due to vasoconstriction or merely the result of an increase in blood viscosity. A cold-induced rise in HLPP was found during saline perfusion, implying that the rise was indeed dependent on vasoconstriction (Figure 3.17). Increased viscosity would also appear to contribute to the rise in HLPP since the slope of the temperature-HLPP curves had a tendency to be lower than that for the blood-perfused hindlimb, though this did not achieve statistical significance (slope = 17.19 ± 4.40 vs. 23.83 ± 4.38 for saline- and blood-perfused respectively; $P$=0.34, unpaired t-test).

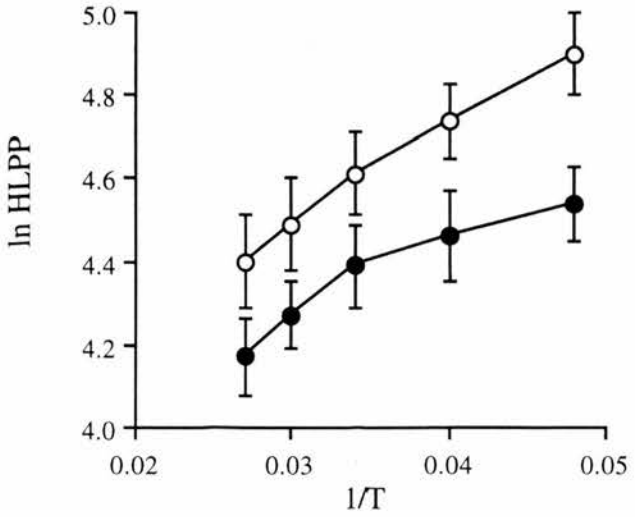



***Figure 3.17.*** *Comparison of the cold-induced increase in hindlimb perfusion pressure (HLPP) in the blood- and saline-perfused hindlimb of anaesthetised rats: temperature (T)-HLPP curves in blood perfused (○; n=6) and saline perfused (●; n=4). All values are mean ± SEM.*

### 3.2.4. Contribution of the cutaneous microcirculation to HLPP

Surgical removal of the skin from the perfused hindlimb did not cause a significant difference in the slope of temperature-HLPP curves (slope = 20.95 ± 10.12 before vs. 20.46 ± 2.82 after removal of hindlimb skin; $P$=0.96, paired t-test) (Figure 3.18). This implied that the skin microcirculation did not contribute to the cold-induced vasoconstriction.

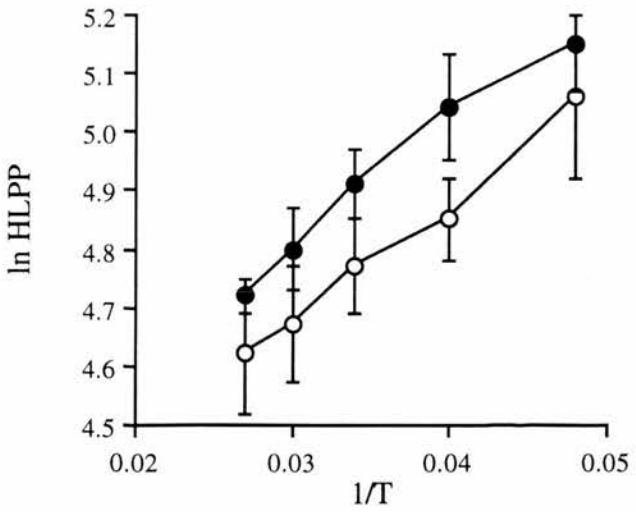



***Figure 3.18.*** *The effect of hindlimb skin removal on the cold-induced increase in hindlimb perfusion pressure (HLPP) in anaesthetised rats: temperature (T)-HLPP curves before (○; n=4) and after surgical removal of the skin (●; n=4). All values are mean ± SEM.*

The fact that the temperature-dependent changes in HLPP were the result of changes in blood flow to skeletal muscle rather than the cutaneous circulation is a weakness when comparing the present model to the situation that occurs in patients with RD, where the skin microcirculation is primarily affected. Thus, although the effects of cooling in the hindlimb are of interest, one must be aware that the relevance of these findings to RD may be limited.

# CHAPTER 4:
# AN INVESTIGATION OF VASOCONSTRICTOR SUBSTANCES IN VITRO

## 4.1. Introduction

Cooling has been shown to potentiate contractions to noradrenaline in canine cutaneous veins (Janssens & Vanhoutte, 1978). Subsequent studies using selective agonists and antagonists of $\alpha_1$- and $\alpha_2$-adrenoceptors have indicated that it is the $\alpha_2$-adrenoceptor-mediated vasoconstriction which is augmented by cooling, probably through an increase in receptor affinity (Flavahan *et al.*, 1985; Ekenvall *et al.*, 1988; Bodelsson *et al.*, 1990; Harker *et al.*, 1990). Contraction induced by 5-hydroxytryptamine (5-HT), acetylcholine, and adenosine phosphate (ATP) is also enhanced by cooling in canine cutaneous veins (Vanhoutte & Shepherd, 1970).

Cooling does not uniformly augment the response to contractile agents. It would appear to depend on the agonist used and the type of vessel under study. Garcia-Villalon *et al.* (1992) demonstrated that the responses to α-adrenoceptor agonists were inhibited during cooling in rabbit ear arteries, through increased availability of nitric oxide (NO), and in femoral arteries, through a decrease in receptor sensitivity. Contraction to potassium chloride (KCl), a direct activator of smooth muscle, is depressed at 24°C in canine cutaneous veins (Vanhoutte & Shepherd, 1970), a finding which has been attributed to a decrease in smooth muscle permeability to calcium ions ($Ca^{2+}$) during cooling (Vanhoutte, 1980). The response to the potent endothelium-derived vasoconstrictor, endothelin-1 (ET-1), has also been shown to be inhibited by cooling in the rabbit ear artery (Monge *et al.*, 1991). In this study the authors suggest that cooling enhances the release and/or activity of the opposing vasodilator NO.

The studies presented in this chapter were undertaken to examine the effect of cooling on the contractile response to ET-1 in resistance arteries obtained from rat mesenteries and from human subcutaneous fat samples taken at surgery. In order to investigate the role of ET-1 in the pathogenesis of Raynaud's disease, concentration-response curves to ET-1 were also generated in resistance arteries taken from gluteal fat biopsies obtained from control subjects and patients with Raynaud's disease. In addition, the

modulatory role of the vascular endothelium on the response to ET-1 was examined, at both 37°C and at 24°C.

The effects of cooling on the contractions induced by the $\alpha_1$-adrenoceptor agonist, phenylephrine (PE), or by KCl were also examined in rat vessels. The contribution of the endothelium to any cold-induced effect was assessed initially by de-endothelialisation, and, where appropriate, the subsequent use of bosentan, a non-selective $ET_{A/B}$ receptor antagonist, and the NO-synthase and cyclooxygenase inhibitors, $N^G$-nitro-L-arginine methyl ester (L-NAME) and indomethacin respectively.

## 4.2. Small vessel arteriograph (perfusion myograph)

### 4.2.1 The effect of cooling on the response to endothelin-1

#### 4.2.1.1. Rat mesenteric resistance arteries

Mean resting lumen diameter, % contraction to PE and % relaxation to ACh did not differ significantly between the group at 37°C and the group at 24°C (Table 4.1).

***TABLE 4.1.*** *Baseline data for vessels studied at 37°C and 24°C*

| | 37°C | 24°C |
|---|---|---|
| LD (μm) | 282 ± 11 | 280 ± 12 |
| % Contraction to PE | 74 ± 1 | 74 ± 1 |
| % Relaxation to ACh | 93 ± 3 | 91 ± 5 |

*LD, resting lumen diameter; PE, phenylephrine ($10^{-5}$M); ACh, acetylcholine ($10^{-6}$M). Values are mean ± SEM for n = 12 vessels at each temperature (total n = 24/18).*

At 37°C, arteries with endothelium removed had a more than 2-fold greater sensitivity to ET-1 than arteries with an intact endothelium ($EC_{50}$ = 2.4 ± 0.4 x $10^{-9}$M intact vs. 1.0 ± 0.2 x $10^{-9}$M denuded; $P<0.05$) (Table 4.2) (Figure 4.1a). Similarly, at 24°C, removal of the endothelium resulted in a 2-fold increase in sensitivity to ET-1 compared to the intact vessel ($EC_{50}$ = 1.4 ± 0.2 x $10^{-9}$M intact vs. 6.3 ± 1.3 x $10^{-10}$M denuded; $P<0.01$) (Table 4.2) (Figure 4.1b). There was a significant increase in the $E_{max}$ for ET-1 after de-endothelialisation, at both 37°C (from 101 ± 2 to 108 ± 1; $P<0.01$) and 24°C (from 101 ± 2 to 108 ± 1; $P<0.05$) (Table 4.3).

When the effect of temperature on arteries was examined, it was seen that cooling to 24°C caused a 1.7-fold increase in sensitivity to ET-1 ($EC_{50}$ = 2.4 ± 0.4 x $10^{-9}$M at 37°C vs. 1.4 ± 0.2 x $10^{-9}$M at 24°C; $P<0.05$) (Table 4.2) (Figure 4.2a). After denudation, this leftward shift of the concentration-response curve to ET-1 was still apparent, but it was only significant at higher concentrations ($10^{-9}$ and 3x$10^{-9}$M; $P<0.05$ and $P<0.001$ respectively), and not for $EC_{50}$ values ($EC_{50}$ = 1.0 ± 0.2 x $10^{-9}$M at 37°C vs. 6.3 ± 1.3 x $10^{-10}$M at 24°C; $P=0.09$) (Table 4.2) (Figure 4.2b).

***Table 4.2.*** *$EC_{50}$ values for ET-1 concentration-response curves in rat mesenteric resistance arteries*

| | Intact | Denuded |
|---|---|---|
| 37°C | $2.4 \pm 0.4 \times 10^{-9}$M | $1.0 \pm 0.2 \times 10^{-9}$M * |
| 24°C | $1.4 \pm 0.2 \times 10^{-9}$M † | $6.3 \pm 1.3 \times 10^{-10}$M ** |

*Values are mean ± SEM for n = 6 vessels in each group. *P < 0.05 ; **P < 0.01 compared to intact vessel. †P < 0.05 compared to 37°C (unpaired t-test).*

***Table 4.3.*** *$E_{max}$ values for ET-1 concentration-response curves in rat mesenteric resistance arteries*

| | Intact | Denuded |
|---|---|---|
| 37°C | 101 ± 2 | 108 ± 1 ** |
| 24°C | 101 ± 2 | 108 ± 1 * |

*Values are mean ± SEM for n = 6 vessels in each group. *P < 0.05 ; **P < 0.01 compared to intact vessel (unpaired t-test).*

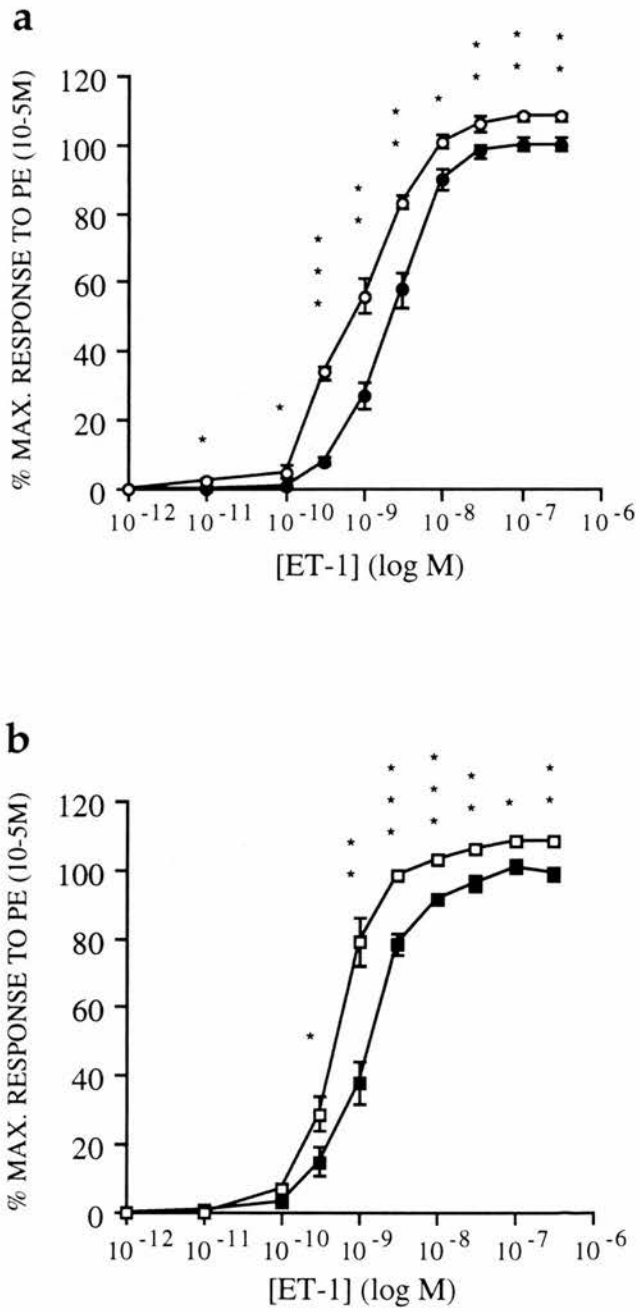



***FIGURE 4.1.*** *The effect of endothelial removal on the contractile response to ET-1 in rat mesenteric resistance arteries: concentration-response curves to ET-1 (expressed as % response to PE, $10^{-5}$M) at 37°C (**Figure 4.1a**), intact (●; n=6) and denuded (○; n=6) and at 24°C (**Figure 4.1b**), intact (■; n=6) and denuded (□; n=6). All values are mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001 (unpaired t-test).*

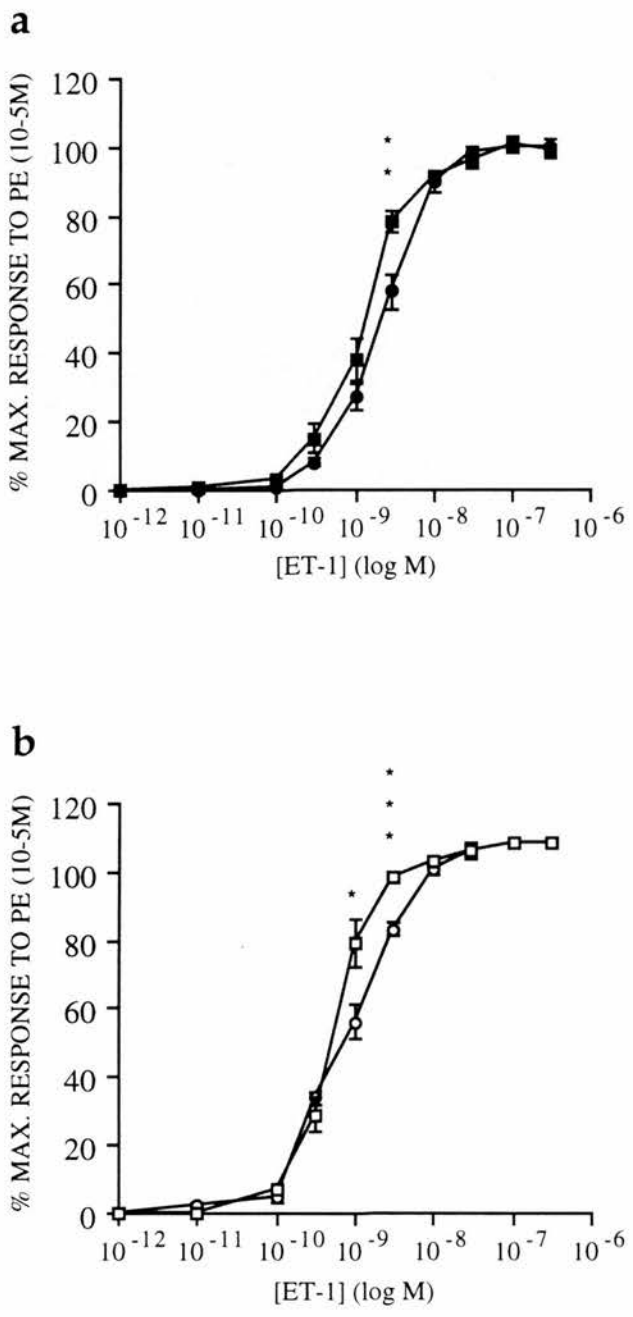



***FIGURE 4.2.*** *The effect of cooling on the contractile response to ET-1 in rat mesenteric resistance arteries: concentration-response curves to ET-1 (expressed as % response to PE, $10^{-5}$M) in intact vessels (**Figure 4.2a**), at 37°C (●; n=6) and at 24°C (■; n=6) and in denuded vessels (**Figure 4.2b**), at 37°C (○; n=6) and at 24°C (□; n=6). All values are mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001 (unpaired t-test).*

In order to further investigate the nature of the endothelium-dependent part of the cold-induced potentiation of the contractile response to ET-1, the effects of the NO synthase inhibitor, $N^G$-nitro-L-arginine methyl ester (L-NAME), and the cyclooxygenase inhibitor, indomethacin, were examined. From Table 4.4, it can be seen that there was no significant difference between mean resting lumen diameter, % contraction to PE, and % relaxation to ACh, for the groups at 37°C and those at 24°C. After a 30 min incubation period, neither L-NAME ($10^{-4}$M) or indomethacin ($10^{-5}$M) significantly changed the resting lumen diameter of the vessels studied (Table 4.4).

***TABLE 4.4.*** *Baseline data for vessels studied at 37°C and 24°C, and the effects of L-NAME or indomethacin on vessel diameter*

| | 37°C | 24°C |
|---|---|---|
| LD | 303 ± 8 | 298 ± 8 |
| % Contraction to PE | 73 ± 1 | 73 ± 0 |
| % Relaxation to ACh | 96 ± 1 | 98 ± 0 |
| LD before L-NAME | 314 ± 16 | 308 ± 15 |
| LD after L-NAME | 315 ± 17 | 285 ± 17 |
| LD before indomethacin | 297 ± 22 | 302 ± 17 |
| LD after indomethacin | 300 ± 24 | 306 ± 18 |

*Resting lumen diameter, LD (μm); phenylephrine, PE ($10^{-5}$M); acetylcholine, ACh ($10^{-6}$M); $N^G$-nitro-L-arginine methyl ester, L-NAME ($10^{-4}$M); indomethacin ($10^{-5}$M). Values are mean ± SEM for n = 11-12 vessels at each temperature (total n = 23/16).*

The % relaxation to ACh was reduced by L-NAME at both 37°C ($P<0.05$) and 24°C ($P<0.001$), but was not significantly altered by indomethacin at either temperature ($P = 0.51$ and $P = 0.36$, at 37°C and 24°C respectively) (Table 4.5). Interestingly, L-NAME tended to produce a greater degree of reduction of the relaxant response to ACh during cooling, compared to that at 37°C, although this difference was not statistically significant ($P = 0.13$, unpaired $t$-test; $P = 0.26$, Wilcoxon rank sum test) (Table 4.5).

***TABLE 4.5.*** *The effects of L-NAME or indomethacin on relaxant response to ACh*

| % Relaxation to ACh: | 37°C | 24°C |
|---|---|---|
| before L-NAME | 94 ± 2 | 99 ± 1 |
| after L-NAME | 37 ± 13 * | 11 ± 9 * * * |
| before indomethacin | 95 ± 3 | 97 ± 1 |
| after indomethacin | 97 ± 1 | 96 ± 1 |

*Acetylcholine, ACh ($10^{-6}$M); $N^{G}$-nitro-L-arginine methyl ester, L-NAME ($10^{-4}$M); indomethacin ($10^{-5}$M). Values are mean ± SEM for n = 5-6 vessels at each temperature (total n= 23/16). *P < 0.05; ***P < 0.001 (paired t-test).*

When combining the control data from this further study (n = 5) with that from the initial study of the effects of cooling on the contractile response to ET-1 (n = 6), it was found that the additional experiments did not increase the significance of the cold-induced leftward shift of the concentration-response curve to ET-1; a 1.8-fold increase in sensitivity was found with n = 11 ($EC_{50}$ = 2.0 ± 0.3 x $10^{-9}$M at 37°C vs. 1.1 ± 0.2 x $10^{-9}$M at 24°C; $P$<0.05) (Table 4.6), compared to 1.7-fold with n = 6 (see above) (Figure 4.3a).

L-NAME ($10^{-4}$M) abolished the cold-induced increase in sensitivity to ET-1 ($EC_{50}$ = 1.3 ± 0.3 x $10^{-9}$M at 37°C vs. 1.3 ± 0.3 x $10^{-9}$M at 24°C; $P$=0.95) (Table 4.6) (Figure 4.3b). The reason for this can be seen when comparing the response to ET-1 in the presence of L-NAME against the control curve at each temperature (Figures 4.4a and 4.4b). Whilst L-NAME induced a leftward-shift of the concentration-response curve to ET-1 at 37°C, no such shift was found at 24°C, thus making the curves superimposible when put together (Figure 4.3b).

In a similar way to endothelial removal in the initial study (Figure 4.2b), the addition of indomethacin prevented a cold-induced shift of the concentration-response curve to ET-1 at lower concentrations, whilst allowing an enhanced sensitivity at higher concentrations, and indeed, an increased maximal response ($E_{max}$ = 103 ± 1 at 37°C vs. 111 ± 2 at 24°C; $P$ < 0.01) (Table 4.7).(Figure 4.3c).

***Table 4.6.*** *$EC_{50}$ values for ET-1 concentration-response curves in rat mesenteric resistance arteries*

| | 37°C | 24°C |
|---|---|---|
| Intact | $2.0 \pm 0.3 \times 10^{-9}$M | $1.1 \pm 0.2 \times 10^{-9}$M |
| L-NAME | $1.3 \pm 0.3 \times 10^{-9}$M | $1.3 \pm 0.3 \times 10^{-9}$M |
| Indomethacin | $2.8 \pm 1.0 \times 10^{-9}$M | $8.9 \pm 0.8 \times 10^{-10}$M |

*$N^G$-nitro-L-arginine methyl ester, L-NAME ($10^{-4}$M); indomethacin ($10^{-5}$M). Values are mean ± SEM for n = 5-6 vessels at each temperature (total n = 23/16).*

***Table 4.7.*** *$E_{max}$ values for ET-1 concentration-response curves in rat mesenteric resistance arteries*

| | 37°C | 24°C |
|---|---|---|
| Intact | 100 ± 1 | 102 ± 1 |
| L-NAME | 107 ± 3 | 107 ± 2 |
| Indomethacin | 103 ± 1 | 111 ± 2 ** |

*$N^G$-nitro-L-arginine methyl ester, L-NAME ($10^{-4}$M); indomethacin ($10^{-5}$M). Values are mean ± SEM for n = 5-6 vessels at each temperature (total n = 23/16). **P < 0.01 (unpaired t-test).*

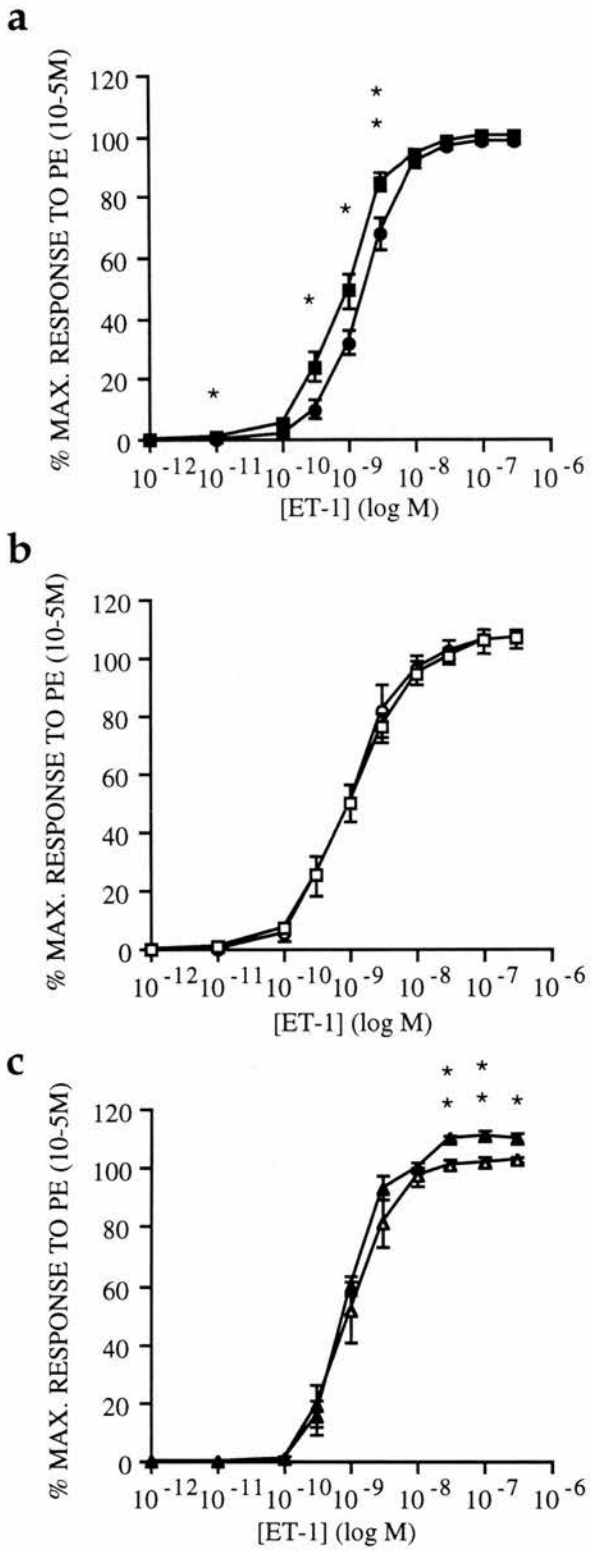



***FIGURE 4.3.*** *The effect of cooling on the contractile response to ET-1 in rat mesenteric resistance arteries: concentration-response curves to ET-1 (expressed as % response to PE, $10^{-5}$M) in: intact vessels (**Figure 4.3a**), at 37°C (●; n=5) and at 24°C (■; n=5); in vessels with L-NAME ($10^{-4}$M) (**Figure 4.3b**), at 37°C (○; n=5) and at 24°C (□; n=6); and in vessels with indomethacin ($10^{-5}$M) (**Figure 4.3c**), at 37°C (Δ; n=6), and at 24°C (▲; n=6). All values are mean ± SEM. *P < 0.05; **P < 0.01 (unpaired t-test).*

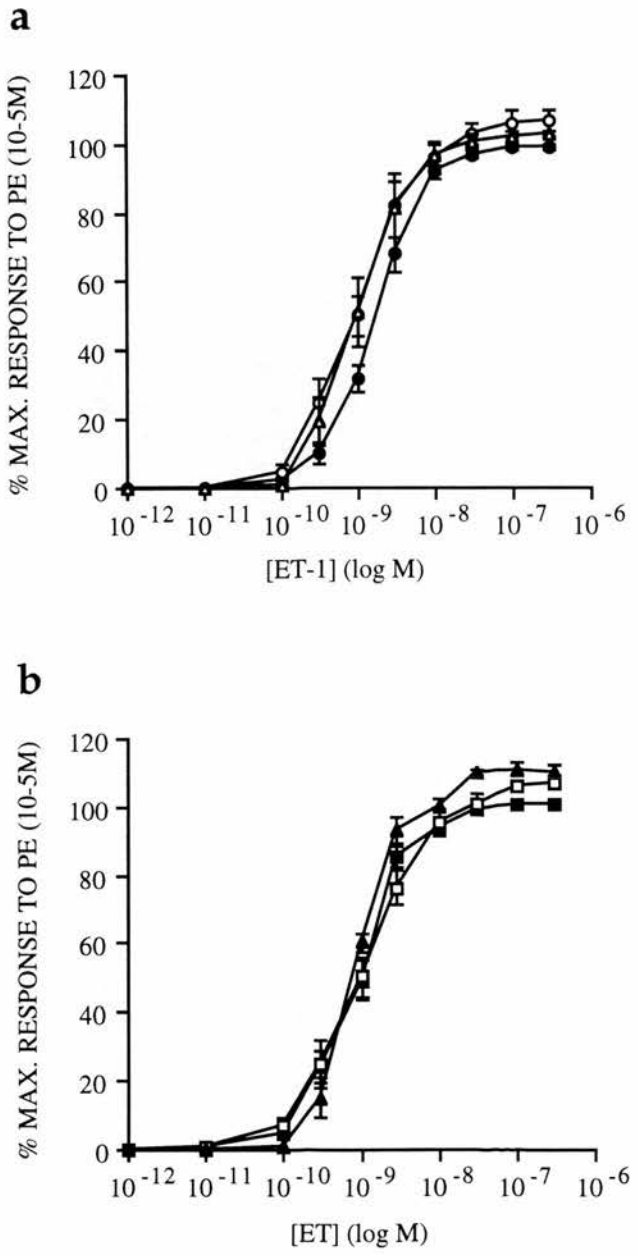



***FIGURE 4.4.*** *The effect of cooling, L-NAME and indomethacin on the contractile response to ET-1in rat mesenteric resistance arteries: concentration-response curves to ET-1 (expressed as % response to PE, $10^{-5}$M) in vessels: at 37°C (**Figure 4.4a**), intact (●; n=5), intact with L-NAME (○; n=5), and intact with indomethacin (Δ; n=6); and at 24°C (**Figure 4.4b**), intact (■; n=5), intact with L-NAME (□; n=6), and intact with indomethacin (▲; n=6). All values are mean ± SEM (for clarity, statistical significance is not shown - see text).*

### 4.2.1.2.Human subcutaneous resistance arteries obtained during surgery

Mean resting lumen diameter, % contraction to KCl and % relaxation to ACh at 37°C did not differ significantly between each group (Table 4.8), and the age and sex ratio of the patients in each group were not significantly different (see Table2.1, Chapter 2).

***TABLE 4.8.*** *Baseline data for vessels studied at 37°C and 24°C*

| | 37°C | 24°C |
|---|---|---|
| LD | 278 ± 26 | 328 ± 56 |
| % Contraction to KCl | 61 ± 6 | 49 ± 7 |
| % Relaxation to ACh | 86 ± 8 | 77 ± 14 |

*Resting lumen diameter, LD (μm); potassium chloride, KCl (60mM); acetylcholine, ACh ($10^{-6}$M). Values are mean ± SEM for n = 8 at 37°C and n = 7 at 24°C.*

ET-1 produced a concentration-dependent constriction in all vessels. Cooling to 24°C caused a decrease in sensitivity to ET-1 as seen by a rightward-shift of the concentration-response curve (Figure 4.5) and an 8-fold increase in the $EC_{50}$ (6.6 ± 2.5 x $10^{-10}$M at 37°C vs. 5.5 ± 2.5 x $10^{-9}$M at 24°C; $P < 0.05$) (Table 4.9). There was a tendency for the maximal contraction to be higher during cooling, but the difference was not statistically significant (114 ± 9 at 37°C vs. 138 ± 14 at 24°C; $P = 0.18$) (Table 4.9).

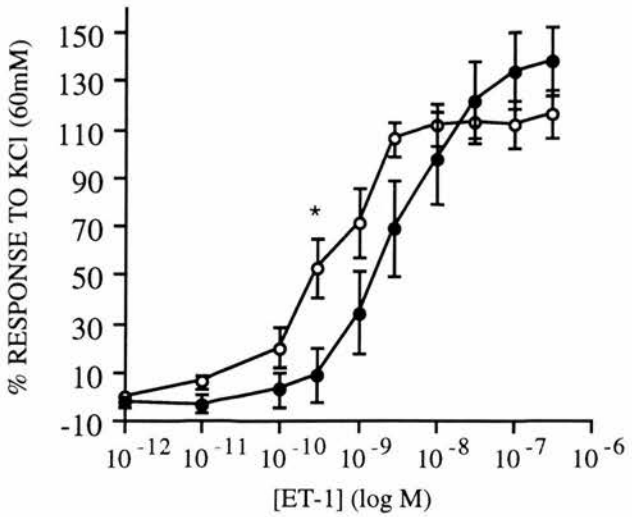



***FIGURE 4.5.*** *The effect of cooling on the contractile response to ET-1 in human subcutaneous resistance arteries: concentration-response curves to ET-1 (expressed as % response to KCl, 60mM) at 37°C (○; n=8) and 24°C (●; n=7). All values are mean ± SEM. *P < 0.05 (unpaired t-test; ANOVA).*

***Table 4.9.*** *$EC_{50}$ and $E_{max}$ values for ET-1 concentration-response curves in human resistance arteries obtained during surgery*

| | 37°C | 24°C |
|---|---|---|
| $EC_{50}$ | $6.6 \pm 2.5 \times 10^{-10}$M | $5.5 \pm 2.5 \times 10^{-9}$M * |
| $E_{max}$ | $114 \pm 9$ | $138 \pm 14$ |

*Values are mean ± SEM for n = 8 at 37°C and n = 7 at 24°C. *P < 0.05 compared to 37°C (unpaired t-test).*

***TABLE 4.10.*** *Mean arterial pressures and blood profiles of control subjects and Raynaud's patients*

| | Normal range | Control subjects | Raynaud's patients |
|---|---|---|---|
| Mean arterial pressure (mmHg) | 85 - 100 | 92 ± 2 | 94 ± 2 |
| Haemoglobin (g/l) | 120 - 180 | 128 ± 3 | 131 ± 3 |
| ESR (mm/hr) | 1 - 9 | 5 ± 1 | 5 ± 1 |
| Urea (mmol/l) | 3.3 - 6.6 | 4.3 ± 0.3 | 4.2 ± 0.3 |
| Sodium (mmol/l) | 135 - 145 | 140 ± 1 | 140 ± 0 |
| Potassium (mmo/l) | 3.5 - 5.0 | 4.7 ± 0.1 | 4.7 ± 0.1 |
| Glucose (mmol/l) | 3.9 - 5.0 | 4.6 ± 0.2 | 4.3 ± 0.2 |
| Creatinine (μmol/l) | 70 - 110 | 75 ± 4 | 71 ± 2 |

*ESR = erythrocyte sedimentation rate. Values are shown as mean ± sem.*

### 4.2.1.3. Human subcutaneous resistance arteries from gluteal biopsies

The mean arterial pressure of control subjects and patients with Raynaud's disease were not significantly different ($P$=0.48) (Table 4.10). Normal haemoglobin, erythrocyte sedimentation rate, urea, electrolytes, glucose and creatinine were confirmed in all volunteers (Table 4.10). The results from the immunological screening showed antinuclear antibodies (ANA) were present in one control subject and four Raynaud's patients.

Mean resting lumen diameter, % contraction to KCl and % relaxation to ACh (for intact artery studies) did not differ significantly between the control subject group, at 37°C and 24°C, and the Raynaud's patient group, at 37°C and 24°C (Table 4.11).

***TABLE 4.11.*** *Baseline data for vessels studied at 37°C and 24°C*

| | Control subjects | | Raynaud's patients | |
|---|---|---|---|---|
| | 37°C | 24°C | 37°C | 24°C |
| LD | 350 ± 37 | 328 ± 20 | 318 ± 26 | 300 ± 21 |
| % Contraction to KCl | 63 ± 4 | 68 ± 3 | 65 ± 2 | 70 ± 2 |
| % Relaxation to ACh | 71 ± 6 | 65 ± 11 | 68 ± 9 | 81 ± 5 |

*Resting lumen diameter, LD (μm); potassium chloride, KCl (60mM); acetylcholine, ACh ($10^{-6}$M). Values are mean ± SEM for n = 12-13 for LD and % contraction to KCl (total n = 49/34), and n= 6-7 for % relaxation to ACh (total = 26/24).*

From Figure 4.6a it can be seen that cooling increased the sensitivity to ET-1 in intact arteries from control subjects ($EC_{50}$ = 3.7 ± 0.7 x $10^{-10}$M at 37°C vs. 1.8 ± 0.3 x $10^{-10}$M at 24°C; $P$<0.05) (Table 4.12). After removal of the endothelium, the sensitivity to ET-1 was reduced at 24°C compared to 37°C ($EC_{50}$ = 1.7 ± 0.3 x $10^{-10}$M at 37°C vs. 6.0 ± 1.8 x $10^{-10}$M at 24°C; $P$<0.05) (Table 4.12) (Figure 4.6b). When comparing the effects of endothelial removal at individual temperatures in control arteries, it was found that denudation enhanced contraction to ET-1 at 37°C

($EC_{50}$ = 3.7 ± 0.7 x $10^{-10}$M in intact arteries vs.1.7 ± 0.3 x $10^{-10}$M in denuded arteries; $P$<0.05) (Table 4.12) (Figure 4.7a), whilst at 24°C it caused a rightward-shift of the concentration-response curve to ET-1 ($EC_{50}$ =1.8 ± 0.3 x $10^{-10}$M in intact arteries vs. 6.0 ± 1.8 x $10^{-10}$M; $P$<0.05) (Table 4.12) (Figure 4.7b). There were no significant changes in maximal contraction to ET-1 after cooling or denudation of vessels from control subjects (Table 4.13).

Cooling did not significantly affect the response to ET-1 in either intact (Figure 4.8a) or denuded vessels obtained from Raynaud's patients (Figure 4.8b) ($EC_{50}$ = 1.9 ± 0.3 x $10^{-10}$M at 37°C vs. 3.0 ± 0.6 x $10^{-10}$M at 24°C in intact arteries; $P$=0.16; and $EC_{50}$ = 4.6 ± 1.2 x $10^{-10}$M at 37°C vs. 4.3 ± 1.0 x $10^{-10}$M at 24°C in denuded arteries; $P$=0.82) (Table 4.14). Cooling had no effect on the maximum contraction to ET-1 in arteries from Raynaud's patients (Table 4.15).

Figure 4.9a shows that removal of the endothelium tended to reduce the sensitivity to ET-1 at 37°C in arteries from patients with Raynaud's disease, although this did not quite reach significance ($EC_{50}$ = 1.9 ± 0.3 x $10^{-10}$M in intact arteries vs. 4.6 ± 1.2 x $10^{-10}$M in denuded arteries; $P$=0.05) (Table 4.14). At 24°C there was no significant difference between intact and denuded arteries ($EC_{50}$ = 3.0 ± 0.6 x $10^{-10}$M in intact arteries vs. 4.3 ± 1.0 x $10^{-10}$M in denuded arteries; $P$=0.30) (Table 4.14) (Figure 4.9b). The maximum contraction to ET-1 did not change significantly after endothelial removal in vessels from Raynaud's patients (Table 4.15).

***TABLE 4.12.*** *$EC_{50}$ values for ET-1 concentration-response curves in resistance arteries obtained from gluteal biopsies from control subjects*

| | Intact | Denuded |
|---|---|---|
| 37°C | $3.7 \pm 0.7 \times 10^{-10}$M | $1.7 \pm 0.3 \times 10^{-10}$M * |
| 24°C | $1.8 \pm 0.3 \times 10^{-10}$M † | $6.0 \pm 1.8 \times 10^{-10}$M *† |

*Values are mean ± SEM for n = 6 vessels in each group. *P < 0.05 compared to intact vessel. †P < 0.05 compared to 37°C (unpaired t-test).*

***TABLE 4.13.*** *$E_{max}$ values for ET-1 concentration-response curves in resistance arteries obtained from gluteal biopsies from control subjects*

| | Intact | Denuded |
|---|---|---|
| 37°C | 117 ± 10 | 124 ± 7 |
| 24°C | 122 ± 5 | 114 ± 3 |

*Values are mean ± SEM for n = 6 vessels in each group.*

***TABLE 4.14.*** *$EC_{50}$ values for ET-1 concentration-response curves in resistance arteries obtained from gluteal biopsies from Raynaud's patients*

| | Intact | Denuded |
|---|---|---|
| 37°C | $1.9 \pm 0.3 \times 10^{-10}$M | $4.6 \pm 1.2 \times 10^{-10}$M |
| 24°C | $3.0 \pm 0.6 \times 10^{-10}$M | $4.3 \pm 1.0 \times 10^{-10}$M |

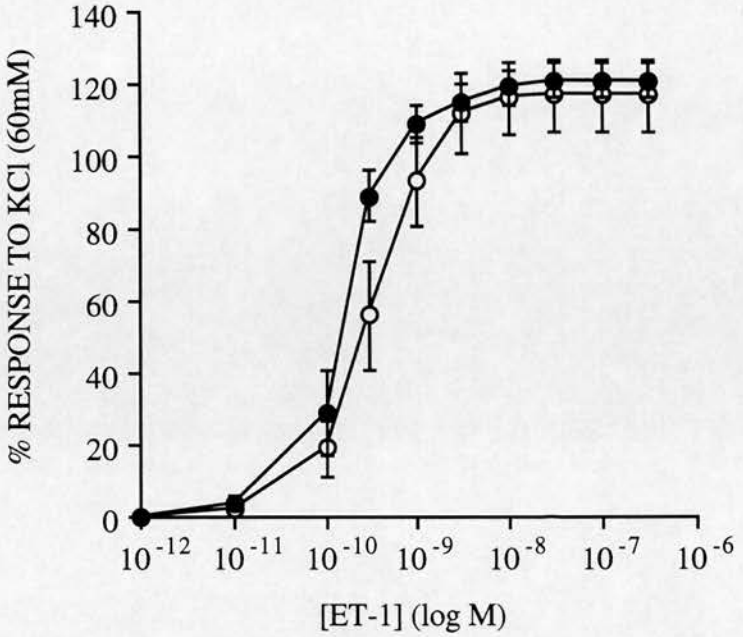
*Values are mean ± SEM for n = 6 vessels in each group.*

***TABLE 4.15.*** *$E_{max}$ values for ET-1 concentration-response curves in resistance arteries obtained from gluteal biopsies from Raynaud's patients*

| | Intact | Denuded |
|---|---|---|
| 37°C | 122 ± 4 | 117 ± 9 |
| 24°C | 124 ± 4 | 120 ± 4 |

*Values are mean ± SEM for n = 6 vessels in each group.*

**a**




**b**

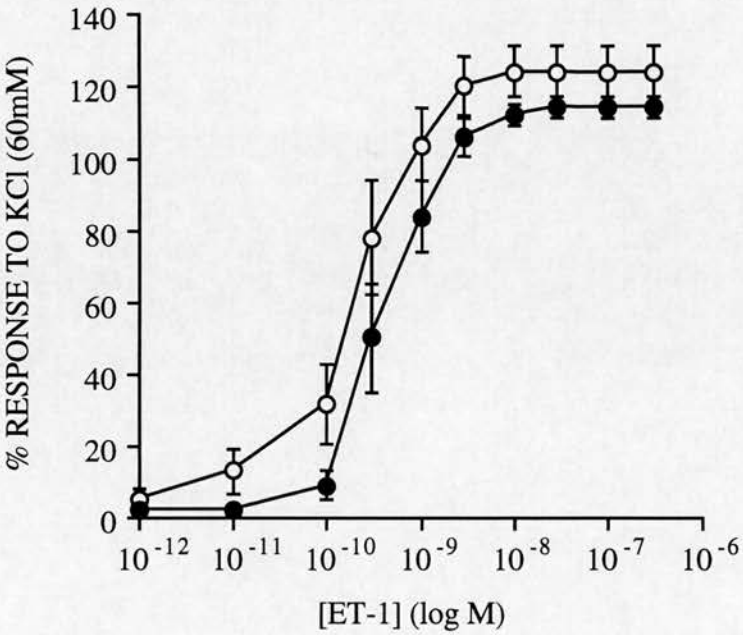
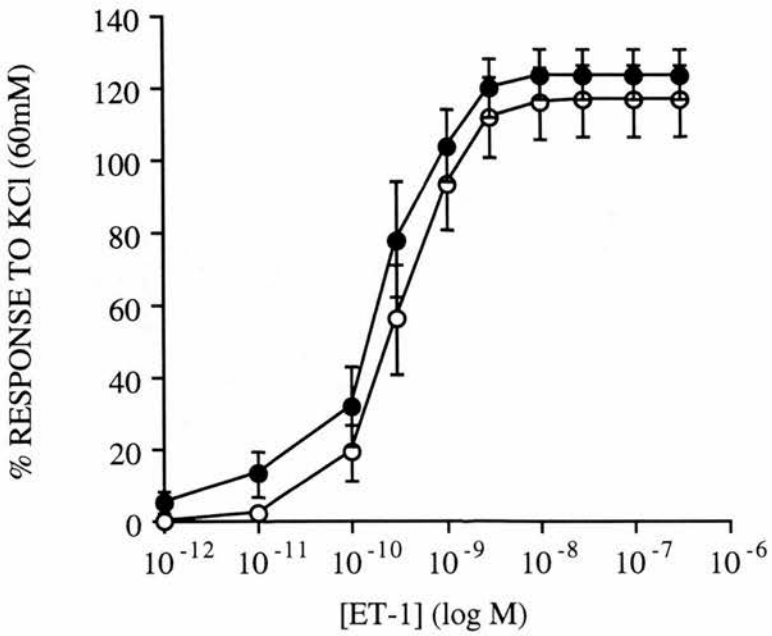



***FIGURE 4.6.*** *The effect of cooling on the contractile response to ET-1 in human subcutaneous resistance arteries from control subjects: concentration-response curves to ET-1 (expressed as % response to KCl, 60mM) in intact vessels (**Figure 4.6a**) and in denuded vessels (**Figure 4.6b**), at 37°C (○; n=6) and at 24°C (●; n=6). All values are mean ± SEM.*

**a**




**b**

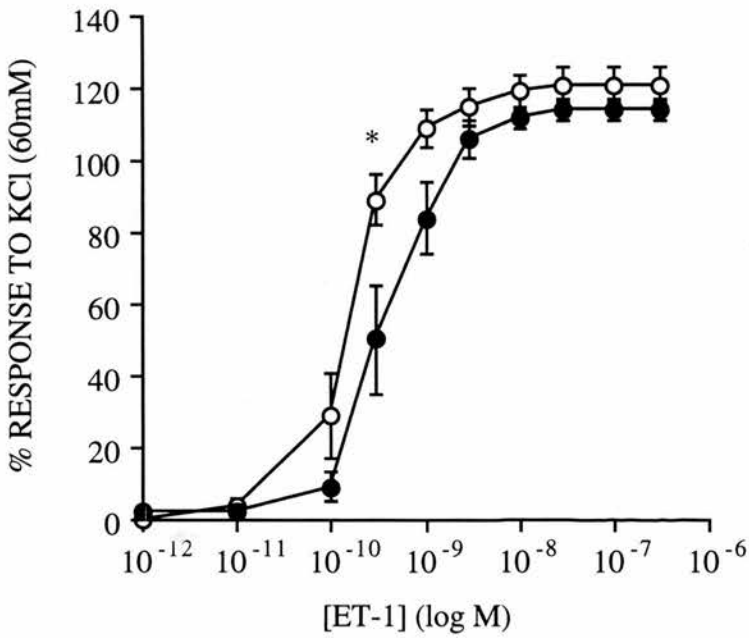
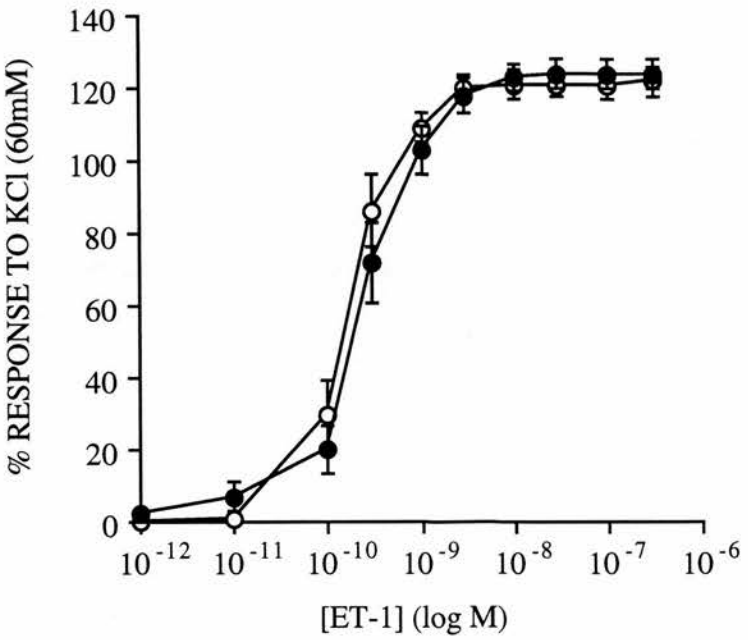



***FIGURE 4.7.*** *The effect of de-endothelialisation on the contractile response to ET-1 in human subcutaneous resistance arteries from control subjects: concentration-response curves to ET-1 (expressed as % response to KCl, 60mM) in vessels at 37°C (**Figure 4.7a**) and at 24°C (**Figure 4.7b**), in intact vessels (○; n=6) and in denuded vessels (●; n=6). All values are mean ± SEM. * P < 0.05 (unpaired t-test; ANOVA).*

**a**




**b**

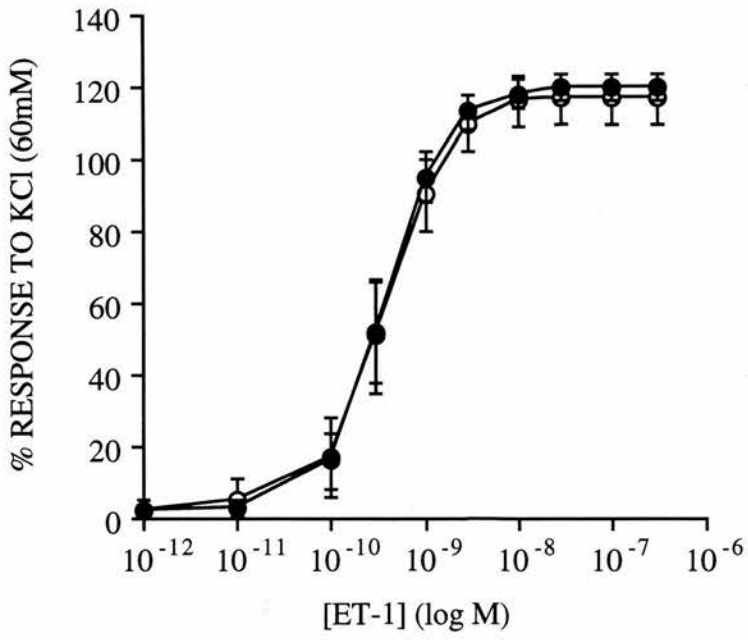
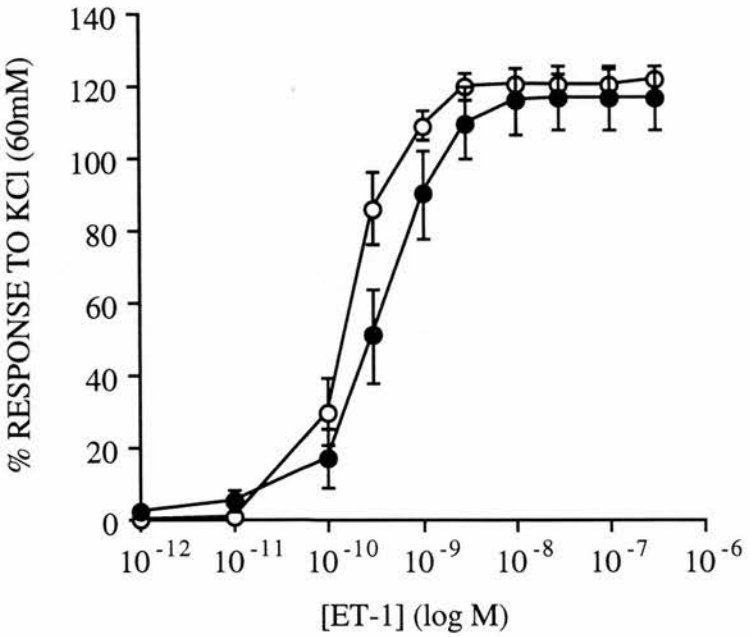



***FIGURE 4.8.*** *The effect of cooling on the contractile response to ET-1 in human subcutaneous resistance arteries from Raynaud's patients: concentration-response curves to ET-1 (expressed as % response to KCl, 60mM) in intact vessels (**Figure 4.8a**) and in denuded vessels (**Figure 4.8b**), at 37°C (○; n=6) and at 24°C (●; n=6). All values are mean ± SEM.*

**a**




**b**

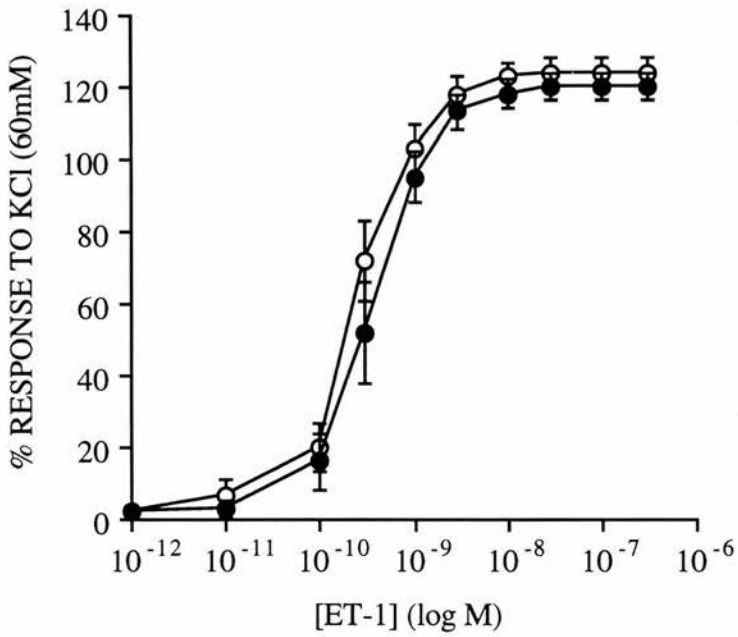



***FIGURE 4.9.*** *The effect of de-endothelialisation on the contractile response to ET-1 in human subcutaneous resistance arteries from Raynaud's patients: concentration-response curves to ET-1 (expressed as % response to KCl, 60mM) in vessels at 37°C (**Figure 4.9a**) and at 24°C (**Figure 4.9b**), in intact vessels (○; n=6) and in denuded vessels (●; n=6). All values are mean ± SEM.*

At 37°C, intact vessels from Raynaud's patients had a higher sensitivity to ET-1 compared to those from control subjects ($EC_{50}$ = 3.7 ± 0.7 x $10^{-10}$M for control group vs. 1.9 ± 0.3 x $10^{-10}$M for Raynaud's group; $P$<0.05) (Table 4.16) (Figure 4.10a) . At 24°C there was no significant difference between arteries from Raynaud's patients and controls ($EC_{50}$ = 1.8 ± 0.3 x $10^{-10}$M for control group vs. 3.0 ± 0.6 x $10^{-10}$M for Raynaud's group; $P$=0.11) (Table 4.16) (Figure 4.10b). The maximal contraction to ET-1 did not differ significantly between each group at either temperature (At 37°C $E_{max}$ = 117 ± 10 for controls vs. 122 ± 4 for Raynaud's ($P$=0.64); and at 24°C $E_{max}$ = 122 ± 5 for controls vs. 124 ± 4 for Raynaud's ($P$=0.75)) (Table 4.17) .

Denuded vessels from Raynaud's patients had a significantly lower sensitivity to ET-1 compared to those from controls at 37°C ($EC_{50}$ = 1.7 ± 0.3 x $10^{-10}$M for control group vs. 4.6 ± 1.2 x $10^{-10}$M for Raynaud's group; $P$<0.05) (Table 4.18) (Figure 4.11a). There was no significant difference between the groups at 24°C ($EC_{50}$ = 6.0 ± 1.8 x $10^{-10}$M for control group vs. 4.3 ± 1.0 x $10^{-10}$M for Raynaud's group; $P$=0.44) (Table 4.18) (Figure 4.11b). At neither temperature was there any significant difference in maximal contraction between the two groups (At 37°C $E_{max}$ = 124 ± 7 for controls vs. 117 ± 9 for Raynaud's; $P$=0.55; at 24°C $E_{max}$ = 114 ± 3 for controls vs. 120 ± 4 for Raynaud's; $P$=0.27) (Table 4.19) .

***TABLE 4.16.*** *$EC_{50}$ values for ET-1 concentration-response curves in* ***intact*** *resistance arteries from control subjects vs. Raynaud's patients*

| | Control | Raynaud's |
|---|---|---|
| 37°C | $3.7 \pm 0.7 \times 10^{-10}$M | $1.9 \pm 0.3 \times 10^{-10}$M * |
| 24°C | $1.8 \pm 0.3 \times 10^{-10}$M | $3.0 \pm 0.6 \times 10^{-10}$M |

*Values are mean ± SEM for n = 6 vessels in each group. *P < 0.05 compared to control vessel (unpaired t-test).*

***TABLE 4.17.*** *$E_{max}$ values for ET-1 concentration-response curves in* ***intact*** *resistance arteries from control subjects vs. Raynaud's patients*

| | Control | Raynaud's |
|---|---|---|
| 37°C | 117 ± 10 | 122 ± 4 |
| 24°C | 122 ± 5 | 124 ± 4 |

*Values are mean ± SEM for n = 6 vessels in each group.*

***TABLE 4.18.*** *$EC_{50}$ values for ET-1 concentration-response curves in* ***denuded*** *resistance arteries from control subjects vs. Raynaud's patients*

| | Control | Raynaud's |
|---|---|---|
| 37°C | $1.7 \pm 0.3 \times 10^{-10}$M | $4.6 \pm 1.2 \times 10^{-10}$M * |
| 24°C | $6.0 \pm 1.8 \times 10^{-10}$M | $4.3 \pm 1.0 \times 10^{-10}$M |

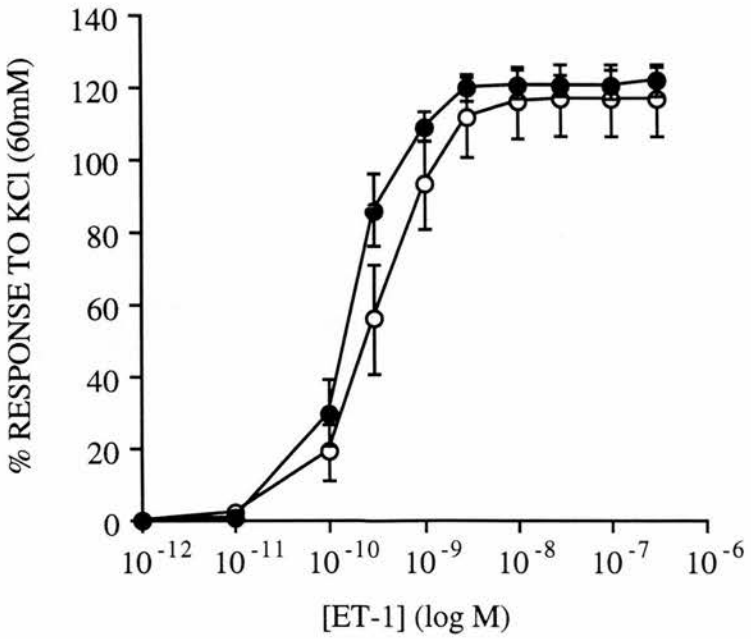
*Values are mean ± SEM for n = 6 vessels in each group. *P < 0.05 compared to intact vessel (unpaired t-test).*

***TABLE 4.19.*** *$E_{max}$ values for ET-1 concentration-response curves in* ***denuded*** *resistance arteries from control subjects vs. Raynaud's patients*

| | Control | Raynaud's |
|---|---|---|
| 37°C | 124 ± 7 | 117 ± 9 |
| 24°C | 114 ± 3 | 120 ± 4 |

*Values are mean ± SEM for n = 6 vessels in each group.*

**a**




**b**

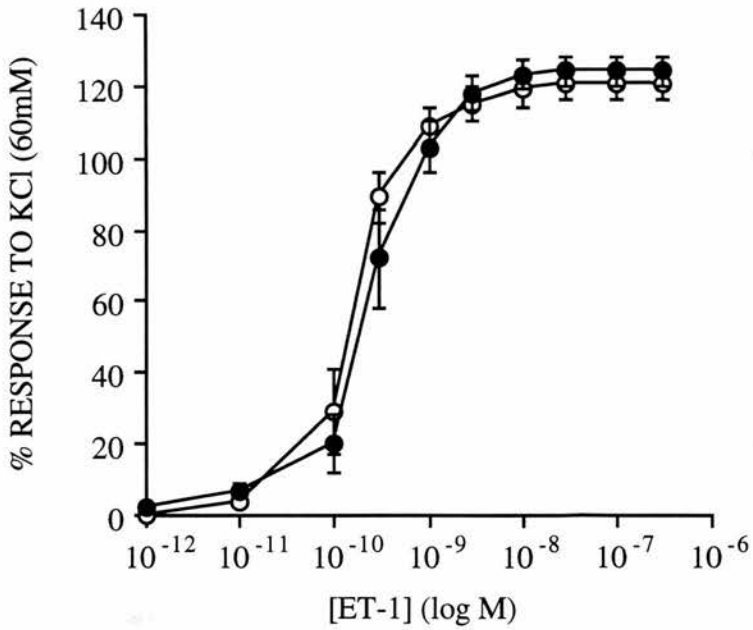
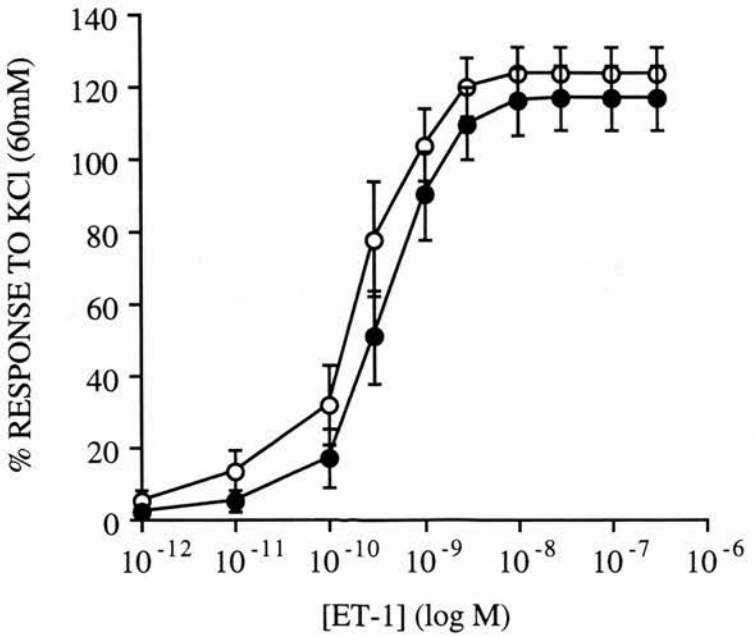



***FIGURE 4.10.*** *A comparison of the contractile response to ET-1 in subcutaneous resistance arteries isolated from control subjects and Raynaud's patients: concentration-response curves to ET-1 (expressed as % response to KCl, 60mM) at 37°C (**Figure 4.10a**) and at 24°C (**Figure 4.10b**), in intact vessels from control subjects (○; n=6) and Raynaud's patients (●; n=6). All values are mean ± SEM.*

**a**




**b**

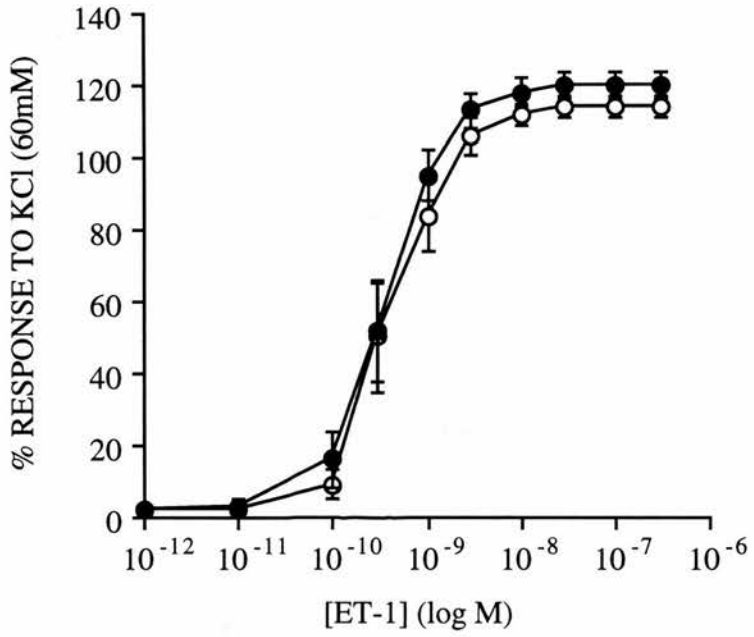



***FIGURE 4.11.*** *A comparison of the contractile response to ET-1 in de-endothelialised resistance arteries from control subjects and Raynaud's patients: concentration-response curves to ET-1 (expressed as % response to KCl, 60mM) at 37°C (**Figure 4.11a**) and at 24°C (**Figure 4.11b**), in denuded vessels from control subjects (○; n=6) and Raynaud's patients (●; n=6). All values are mean ± SEM.*

### 4.2.2. The effect of cooling and antagonism of ET receptors on the response to phenylephrine

#### 4.2.2.1. Rat mesenteric resistance arteries

Mean resting lumen diameter, % contraction to PE and % relaxation to ACh did not differ significantly between the group at 37°C and the group at 24°C (Table 4.20). The presence of bosentan ($10^{-6}$M) had no significant affect on resting lumen diameter (Table 4.20).

***TABLE 4.20.*** *Baseline data for vessels studied at 37°C and 24°C, and the effects of bosentan on vessel diameter*

| | 37°C | 24°C |
|---|---|---|
| LD (μm) | 308 ± 8 | 310 ± 10 |
| % Contraction to PE | 73 ± 1 | 73 ± 1 |
| % Relaxation to ACh | 94 ± 2 | 95 ± 2 |
| LD before bosentan | 323 ± 13 | 323 ± 24 |
| LD after bosentan | 323 ± 13 | 323 ± 24 |

*Resting lumen diameter, LD; phenylephrine, PE ($10^{-5}$M); acetylcholine, ACh ($10^{-6}$M). Values are mean ± SEM for n = 18/18 at 37°C, n= 17/17 at 24°C, and n = 6 for vessels with bosentan ($10^{-6}$M) at 37°C and 24°C (total = 12/7).*

Figure 4.12 shows the effect of the mixed $ET_{A/B}$-receptor antagonist, bosentan ($10^{-6}$M), against ET-1. There was a rightward-shift of the concentration-response curve to ET-1, confirming that the same dose used in the PE experiments was sufficient to antagonise the effects of ET ($EC_{50}$ values were not calculated because maximal contraction to ET-1 was not obtained in the presence of bosentan).

In arteries with an intact endothelium, cooling to 24°C caused a 4-fold increase in sensitivity to PE ($EC_{50}$ = 5.9 ± 1.0 $10^{-7}$M at 37°C vs. 1.4 ± 0.2 x $10^{-7}$M at 24°C; $P<0.01$) (Table 4.21) (Figure 4.13a). After denudation, this leftward-shift of the concentration-response curve to PE was still apparent ($EC_{50}$ = 5.7 ± 1.0 $10^{-7}$M at 37°C vs. 2.9 ± 5.0 x $10^{-7}$M at 24°C; $P=0.05$) (Table 4.21) (Figure 4.13b), but was significantly reduced compared to the intact arteries at 24°C ($P<0.05$). Similarly, the presence of bosentan ($10^{-6}$M) reduced the cold-induced increase in sensitivity such

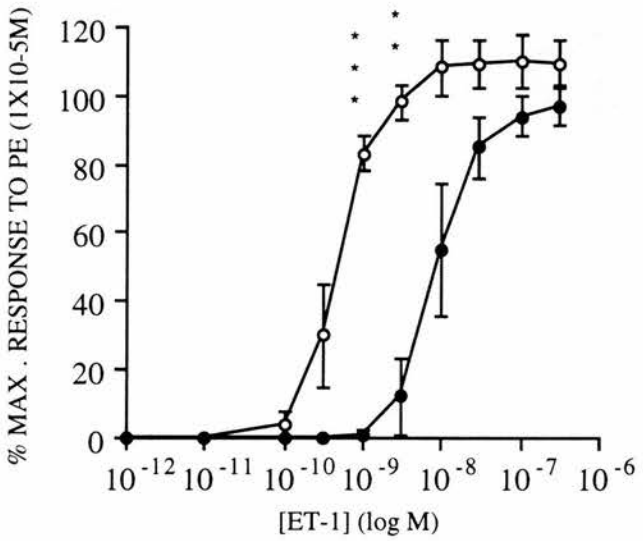



***FIGURE 4.12.*** *The antagonist action of bosentan on the contractile response to ET-1 in rat mesenteric resistance arteries: concentration-response curves to ET-1 (expressed as % response to PE, $10^{-5}$M) in control vessels (○; n=3) and in vessels with bosentan ($10^{-6}$M) present (●; n=3) at 37°C. All values are mean±SEM. **P<0.01; ***P<0.001.*

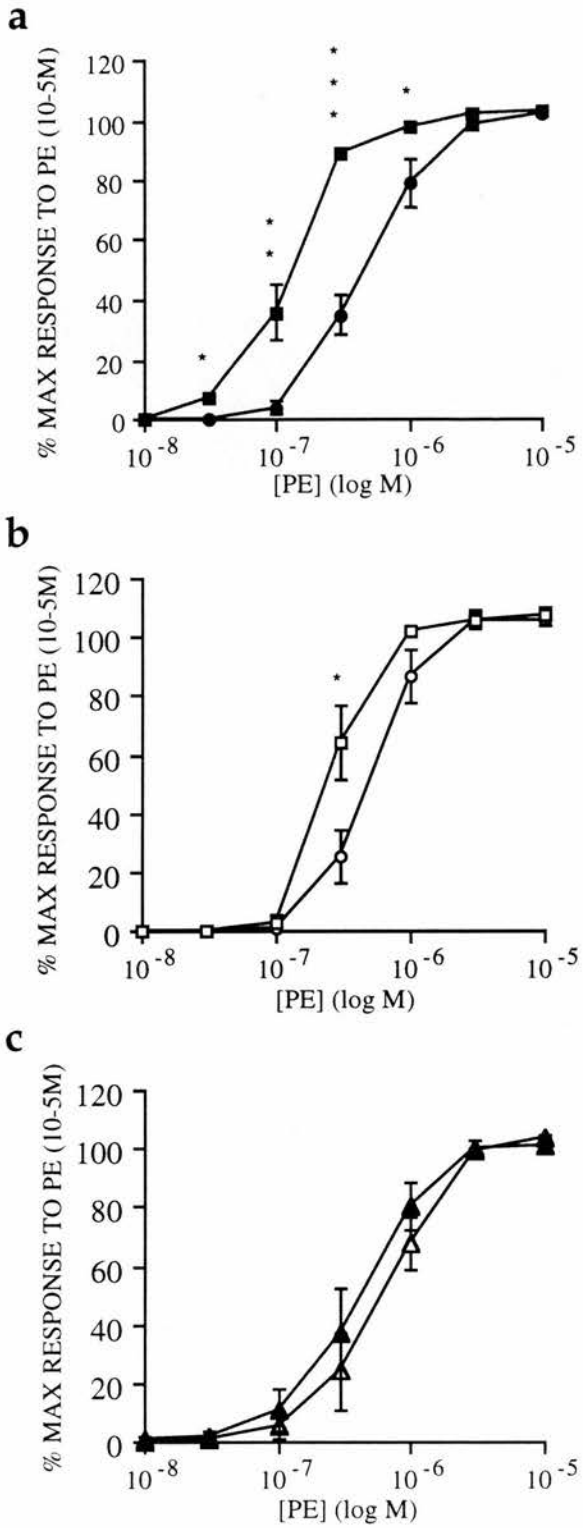



***FIGURE 4.13.*** *The effect of cooling on the contractile response to PE in rat mesenteric resistance arteries: concentration-response curves to PE (expressed as % response to 'wake-up' PE, $10^{-5}$M) in: intact vessels (**Figure 4.13a**), at 37°C (●; n=6) and at 24°C (■; n=6); in denuded vessels (**Figure 4.13b**), at 37°C (○; n=6) and at 24°C (□; n=6); and in intact vessels with bosentan present (**Figure 4.13c**), at 37°C (Δ; n=6) and at 24°C (▲; n=6). All values are mean ± SEM. *P<0.05; **P<0.01; ***P<0.001.*

that the leftward-shift of the concentration-response curve to PE no longer reached significance ($EC_{50}$ = 7.6 ± 1.6 $10^{-7}$M at 37°C vs. 4.9 ± 1.2 x $10^{-7}$M at 24°C; $P$=0.34) (Table 4.21) (Figure 4.13c).

When combining the above results for each temperature, it was seen that at 37°C endothelial removal or bosentan ($10^{-6}$M) had no significant effect on the sensitivity of arteries to PE, compared to intact control vessels ($EC_{50}$ = 5.9 ± 1.0 x $10^{-7}$M intact vs. 5.7 ± 1.0 x $10^{-7}$M denuded ($P$=0.88), and vs. 7.6 ± 1.6 x $10^{-7}$M with bosentan ($P$=0.39)) (Table 4.21) (Figure 4.14a). However, at 24°C, removal of the endothelium or addition of bosentan resulted in a 2- ($EC_{50}$ = 1.4 ± 0.2 x $10^{-7}$M intact vs.2.9 ± 5.0 x $10^{-7}$M denuded; $P$<0.05) and 3-($EC_{50}$ = 1.4 ± 0.2 x $10^{-7}$M intact vs. 4.9 ± 1.2 x $10^{-7}$M with bosentan; $P$<0.05) fold decrease in sensitivity to PE, respectively, compared to the intact control vessel (Table 4.21) (Figure 4.14b). There was no significant difference in the $E_{max}$ for PE between any of the groups studied (Table 4.22).

***TABLE 4.21.*** *$EC_{50}$ values for PE concentration-response curves in rat mesenteric resistance arteries at 37°C and 24°C*

| | 37°C | 24°C |
|---|---|---|
| Intact (n=6-7) | 5.9 ± 1.0 x $10^{-7}$M | 1.4 ± 0.2 x $10^{-7}$M ** |
| Denuded (n=5-6) | 5.7 ± 1.0 x $10^{-7}$M | 2.9 ± 5.0 x $10^{-7}$M |
| Bosentan (n=6) | 7.6 ± 1.6 x $10^{-7}$M | 4.9 ± 1.2 x $10^{-7}$M |

*Bosentan, mixed $ET_A/ET_B$ receptor antagonist ($10^{-6}$M). Values are mean ± SEM. n = number of vessels. **P < 0.01 compared to 37°C (unpaired t-test).*

***TABLE 4.22.*** *$E_{max}$ values for PE concentration-response curves in rat mesenteric resistance arteries at 37°C and 24°C*

| | 37°C | 24°C |
|---|---|---|
| Intact (n=6-7) | 103 ± 1 | 103 ± 1 |
| Denuded (n=5-6) | 106 ± 2 | 107 ± 3 |
| Bosentan (n=6) | 104 ± 1 | 101 ± 3 |

*Bosentan, mixed $ET_A/ET_B$ receptor antagonist ($10^{-6}$M). Values are mean ± SEM. n = number of vessels.*

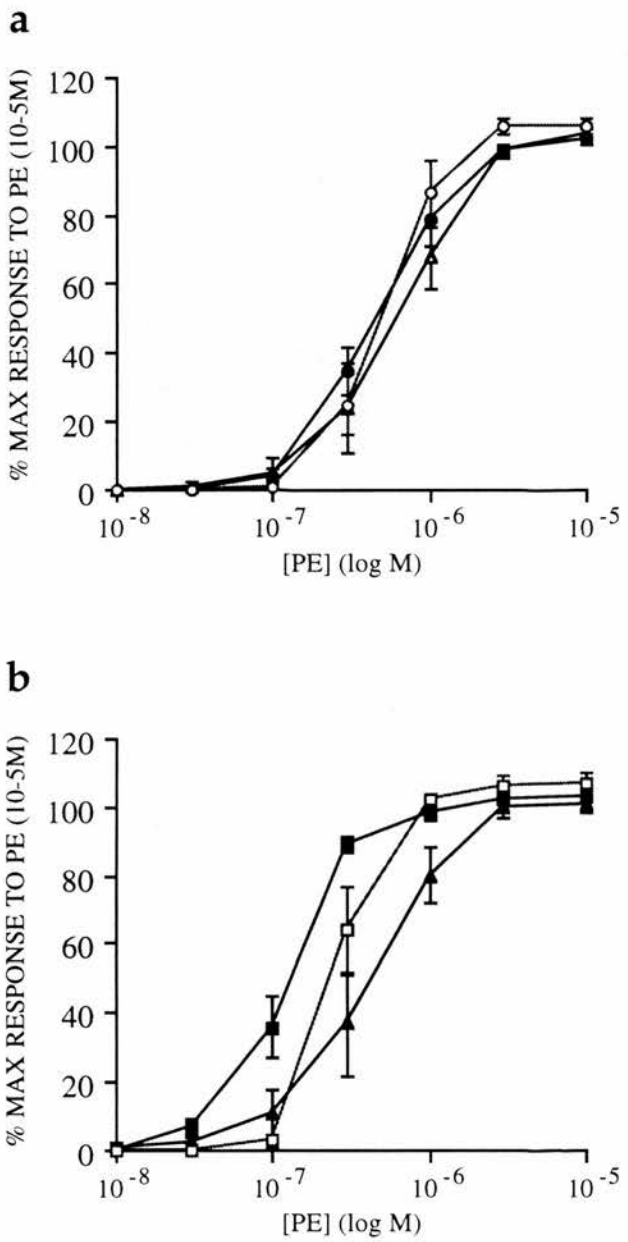



***FIGURE 4.14.*** *The effect of cooling, removal of the endothelium, and bosentan on the contractile response to PE in rat mesenteric resistance arteries: concentration-response curves to PE (expressed as % response to 'wake-up' PE, $10^{-5}$M) in vessels: at 37°C (**Figure 4.14a**), intact (●; n=6), denuded (○; n=6), and intact with bosentan (Δ; n=6); and at 24°C (**Figure 4.14b**), intact (■; n=6), denuded (□; n=6), and intact with bosentan present (▲; n=6). All values are mean ± SEM (for clarity, statistical significance is not shown - see text).*

### 4.2.3. The effect of cooling on the response to potassium chloride

#### 4.2.3.1. Rat mesenteric resistance arteries

Mean resting lumen diameter, % contraction to PE and % relaxation to ACh did not differ significantly between the group at 37°C and the group at 24°C (Table 4.23). The presence of combined L-NAME ($10^{-4}$M) and indomethacin ($10^{-5}$M) did not cause a significant change in resting lumen diameter (Table 4.23). The % relaxation to ACh was not significantly attenuated after the addition of L-NAME and indomethacin (Table 4.24).

***TABLE 4.23.*** *Baseline data for vessels studied at 37°C and 24°C, and the effects of L-NAME/indomethacin on vessel diameter*

| | 37°C | 24°C |
|---|---|---|
| LD | 298 ± 4 | 309 ± 6 |
| % Contraction to PE | 73 ± 1 | 73 ± 0 |
| % Relaxation to ACh | 98 ± 1 | 95 ± 1 |
| LD before L-NAME/indo | 290 ± 12 | 310 ± 8 |
| LD after L-NAME/indo | 292 ± 12 | 310 ± 8 |

*Resting lumen diameter, LD (μm); phenylephrine, PE ($10^{-5}$M); acetylcholine, ACh ($10^{-6}$M); $N^G$-nitro-L-arginine methyl ester, L-NAME ($10^{-4}$M); indomethacin, indo ($10^{-5}$M). Values are mean ± SEM for n = 18 at 37°C, n = 21 at 24°C (total n = 39/24) and n = 6 for vessels with L-NAME/indomethacin (total = 12/10).*

***TABLE 4.24.*** *The effects of L-NAME/indomethacin on relaxant response to ACh*

| % Relaxation to ACh: | 37°C | 24°C |
|---|---|---|
| before L-NAME/indo | 98 ± 1 | 98 ± 1 |
| after L-NAME/indo | 86 ± 5 | 84 ± 7 |

*Acetylcholine, ACh ($10^{-6}$M); $N^G$-nitro-L-arginine methyl ester, L-NAME ($10^{-4}$M); indomethacin, indo ($10^{-5}$M). Values are mean ± SEM for n = 6 vessels at each temperature (total n = 12/10).*

In arteries with an intact endothelium, cooling to 24°C decreased the maximal contraction to KCl (Table 4.26), but did not significantly affect the sensitivity ($EC_{50}$ = 26 ± 2 mM at 37°C vs. 21 ± 2 mM at 24°C; $P$=0.06) (Table 4.25) (Figure 4.15a). After denudation, there was no longer a depression of the maximum response to KCl at 24°C (Table 4.26), and cooling in fact caused a marked leftward-shift of the

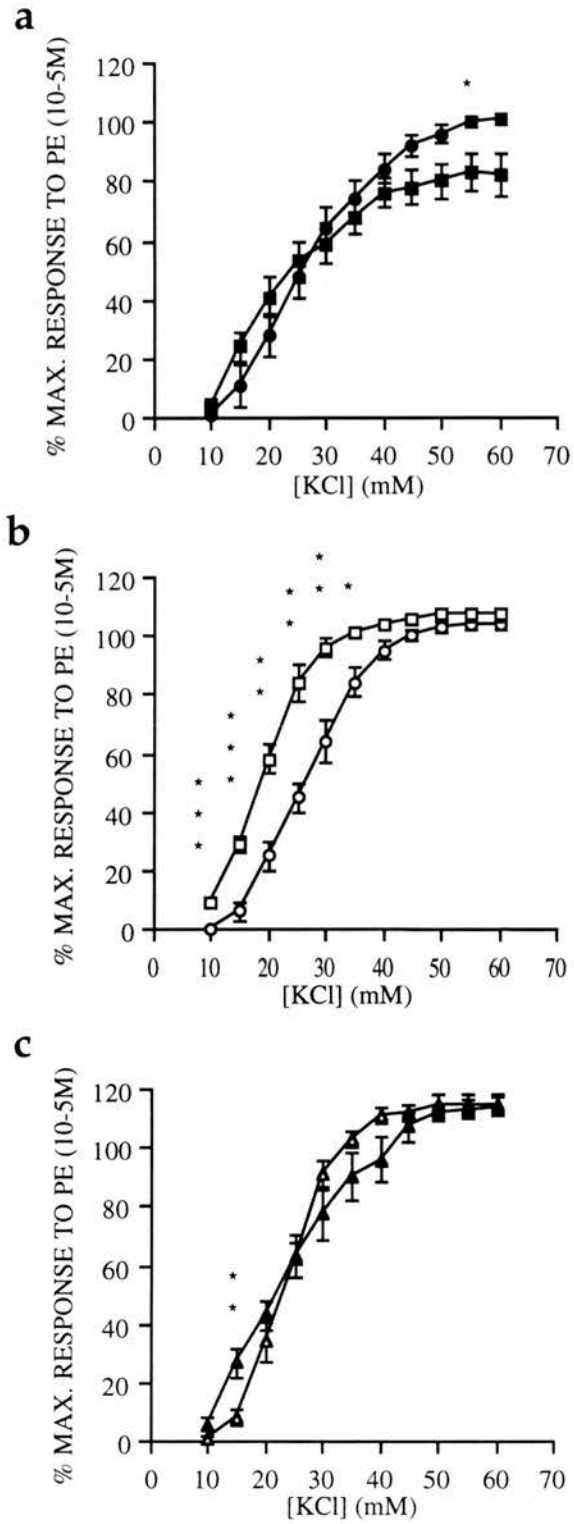



***FIGURE 4.15.*** *The effect of cooling on the contractile response to KCl in rat mesenteric resistance arteries: concentration-response curves to KCl (expressed as % response to PE, $10^{-5}$M) in: intact vessels (**Figure 4.15a**), at 37°C (●; n=6) and at 24°C (■; n=10); in denuded vessels (**Figure 4.15b**), at 37°C (○; n=6) and at 24°C (□; n=5); and in intact vessels with L-NAME ($10^{-4}$M) and indomethacin ($10^{-5}$M) present (**Figure 4.15c**), at 37°C (Δ; n=6) and at 24°C (▲; n=6). All values are mean ± SEM. *P<0.05; **P<0.01; ***P<0.001.*

concentration-response curve to KCl ($EC_{50}$ = 27 ± 1 mM at 37°C vs. 20 ± 1 mM at 24°C; $P<0.01$) (Table 4.25) (Figure 4.15b). Similarly, the combination of L-NAME and indomethacin restored the maximum contractile response to KCl during cooling (Table 4.26), and caused a slight leftward-shift of the concentration-response curve, but only at low concentrations of KCl (Figure 4.15c). In the presence of L-NAME and indomethacin there was no significant change to $EC_{50}$ values during cooling ($EC_{50}$ = 24 ± 1 mM at 37°C vs. 24 ±1 mM at 24°C; $P=0.93$) (Table 4.25).

When combining the above results for each temperature, it was seen that at 37°C removal of the endothelium had no significant effect on the sensitivity of arteries to KCl, in comparison with intact control vessels ($EC_{50}$ = 26 ± 2 mM intact vs. 27 ± 1 mM denuded; $P=0.50$) (Table 4.25) (Figure 4.16a). The concentration-response curve to KCl in the presence of L-NAME and indomethacin lay to the left of that for intact arteries, but the leftward-shift failed to reach significance ($EC_{50}$ = 26 ± 2 mM intact vs. 24 ± 1 mM with L-NAME/indomethacin; $P=0.45$) (Table 4.25). The maximum contractile response to KCl was not significantly affected by endothelial removal at 37°C, but L-NAME and indomethacin significantly enhanced the response (Table 4.26). At 24°C, the sensitivity to KCl was unaffected by removal of the endothelium ($EC_{50}$ = 21 ± 2 mM intact vs. 20 ± 1 mM denuded; $P=0.46$) (Table 4.25) or by the addition of L-NAME and indomethacin ($EC_{50}$ = 22 ± 2 mM intact vs. 24 ± 1 mM with L-NAME/indomethacin; $P=0.31$) (Table 4.25) (Figure 4.16b). Maximal contraction to KCl was significantly increased with endothelial removal or addition of L-NAME and indomethacin (Table 4.26).

***TABLE 4.25.*** *$EC_{50}$ values for KCl concentration-response curves in rat mesenteric resistance arteries at 37°C and 24°C*

| | 37°C | 24°C |
|---|---|---|
| Intact (n=6-10) | 26 ± 2 mM | 21 ± 2mM |
| Denuded (n=5-6) | 27 ± 1 mM | 20 ± 1 mM ** |
| L-NAME/indo (n=6) | 24 ± 1 mM | 24 ± 1 mM |

*L-NAME ($10^{-4}$M) / indomethacin ($10^{-5}$M). Values are mean ± SEM. n = number of vessels. **P<0.01 compared to 37°C (unpaired t-test).*

***TABLE 4.26.*** *$E_{max}$ values for KCl concentration-response curves in rat mesenteric resistance arteries at 37°C and 24°C*

| | 37°C | 24°C |
|---|---|---|
| Intact (n=6-10) | 101 ± 2 | 83 ± 6 * |
| Denuded (n=5-6) | 104 ± 2 | 107 ± 2 † |
| L-NAME/indo (n=6) | 115 ± 3 † † | 114 ± 3 † † |

*L-NAME ($10^{-4}$M) / indomethacin ($10^{-5}$M). Values are mean ± SEM. n = number of vessels. †P<0.05; ††P<0.01 compared to intact vessel for individual temperature group. *P<0.05 between groups at 37°C and 24°C (unpaired t-test).*

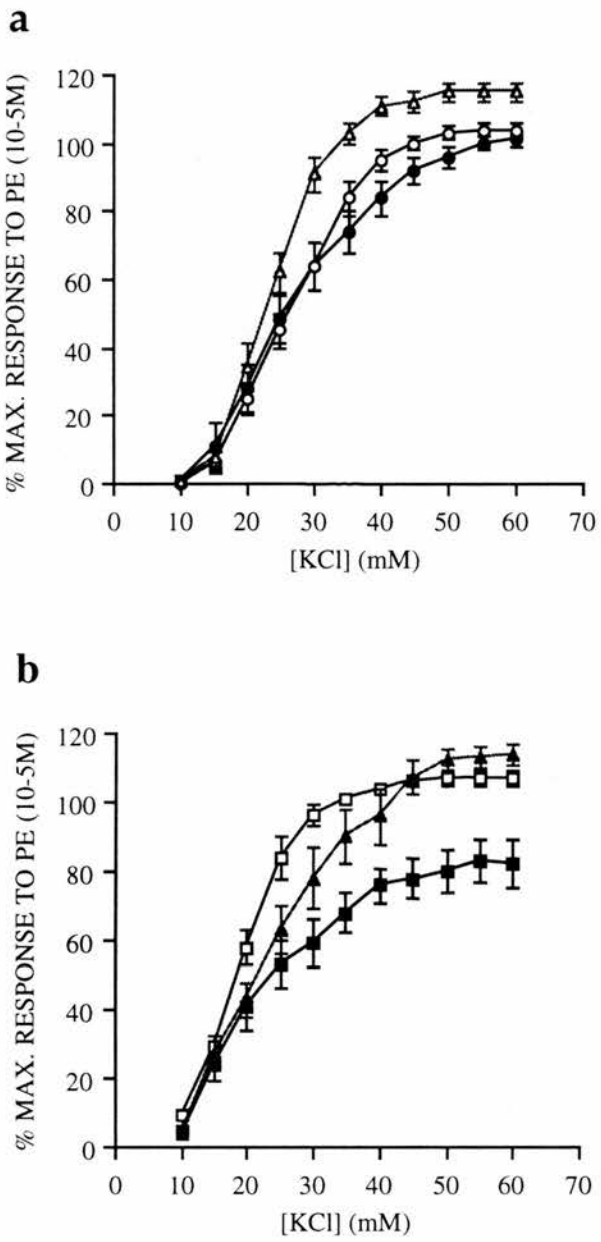



***FIGURE 4.16.*** *The effect of cooling, removal of the endothelium, and L-NAME/indomethacin on the contractile response to KCl in rat mesenteric resistance arteries: concentration-response curves to KCl (expressed as % response to PE, $10^{-5}$M) in vessels: at 37°C (**Figure 4.16a**), intact (●; n=6), denuded (○; n=6), and intact with L-NAME/indomethacin (Δ; n=6); and at 24°C (**Figure 4.16b**), intact (■; n=10), denuded (□; n=5), and intact with L-NAME/indomethacin present (▲; n=6). All values are mean ± SEM (for clarity, statistical significance is not shown - see text).*

## 4.3. Discussion

The results from these studies show that the sensitivity of rat mesenteric resistance arteries to ET-1 is increased during cooling. This is in part due to altered availability and/or actions of endothelium-derived relaxing and contracting factors. Enhanced sensitivity of the vascular smooth muscle to ET-1 also contributes, but this only occurs at high concentrations of ET-1 (nanomolar range) - with regard to normal circulating levels of ET, which in healthy humans are in the low picomolar range (Davenport *et al.*, 1990) this may not appear physiologically relevant, but the concentrations at the endothelial-smooth muscle interface are likely to be much higher than those found in the plasma. Further studies using the cyclooxygenase inhibitor indomethacin revealed that constrictor prostanoids, such as $TXA_2$, probably account for the endothelium-dependent cold-induced leftward shift of the concentration response curve to ET-1.

The experiments carried out in de-endothelialised vessels demonstrate that endothelium-derived dilator substances oppose vasoconstriction to ET-1 at both 37°C *and* 24°C, as seen by enhanced sensitivity and contraction to ET-1 after denudation. Depending on the extent to which NO contributes to vasodilator function in these vessels, one may expect to find a similar increase in the response to ET-1 if NO synthesis is blocked, as indeed was found at 37°C in the presence of the NO synthase inhibitor, L-NAME. Interestingly however, L-NAME had no effect on the response to ET-1 at 24°C, implying that NO is not released during cooling. Although this could account for the enhanced sensitivity to ET-1 at 24°C in intact arteries, it is inconsistent with the results found in denuded vessels which show that endothelium-derived dilator substances oppose ET-1-induced vasoconstriction. A possible explanation for this finding is that denudation removes a factor(s) in addition to NO, such as endothelium-derived hyperpolarising factor (EDHF). Nagao & Vanhoutte (1993) propose that EDHF accounts for a greater proportion of endothelium-dependent dilatation than NO in small arteries, and

clearly, in the present study L-NAME was unable to completely block the relaxant response to acetylcholine (ACh). $PGI_2$ is unlikely to be involved since indomethacin failed to attenuate the dilator response to ACh. Larger vessels have been shown to produce more $PGI_2$ than the microvasculature (MacIntyre *et al.*, 1978), thus $PGI_2$ may have a lesser role in vasodilator function than NO or EDHF in resistance arteries.

Cooling was found to decrease the sensitivity to ET-1 in intact human resistance arteries isolated from subcutaneous fat biopsies obtained during surgery. In a similar study using the rabbit ear artery, Monge *et al.* (1991) examined the effect of the NO synthase inhibitor, L-NAME, and also de-endothelialisation, on responses to ET-1 at 37°C and 24°C. Their findings suggest that the decrease in ET-1 sensitivity during cooling is caused either by an increased availability of NO, which could be due to increased generation or reduced clearance, or, alternatively, due to increased sensitivity to NO. An alternative explanation for these results is a decrease in vascular smooth muscle sensitivity or responsiveness to ET-1 at 24°C.

In the studies using arteries obtained from gluteal fat biopsies, cooling was found to increase the sensitivity to ET-1 in control subjects. After endothelial removal, the sensitivity to ET-1 was reduced during cooling, implying that vasoconstrictor factors are released from the endothelium, which enhances the contraction to ET-1 at 24°C. This is confirmed when the responses to ET-1 are compared between intact and denuded vessels at each temperature. At 37°C, removal of the endothelium increased sensitivity to ET-1, whilst at 24°C it decreased the sensitivity. It would appear, therefore, that the vascular endothelium can modulate the response to ET-1 in different ways in a temperature-dependent manner; namely by releasing dilator substances which oppose vasoconstriction at 37°C, and by releasing constrictor substances which augment vasoconstriction at 24°C. Vasoconstriction in response to moderate cooling is a physiological phenomenon (Vanhoutte, 1980) and the present results are in accord

with this. There are several reasons why the results from the surgical samples are not in agreement with those from the gluteal biopsy studies. Most importantly is probably the fact that the sex ratio and the mean age of the patients are different between the two studies: the majority of surgical patients were male, whilst the biopsy patients were predominantly female and significantly younger. Sex hormones are known to influence vascular reactivity (Williams *et al.* 1988; Yue *et al.*, 1995). When comparing the results from the two studies in light of the difference in the sex ratios, one may propose that in men cooling decreases ET-1-mediated contraction, whilst in women, cooling enhances the response to ET-1. Although very speculative, this suggestion is certainly compatible with the fact that Raynaud's disease affects predominantly females. Another reason for the observed difference in the results between the two studies is that one group comprises patients undergoing gastrointestinal (GI) surgery, whilst the other comprises healthy volunteers. Although not cardiovascular in origin, the conditions requiring surgery, and indeed the medication received, may well affect vascular function in these individuals. In addition, as mentioned in a previous section, volatile anaesthetics can inhibit endothelium-dependent dilatation (Muldoon *et al.*, 1988; Stone & Johns, 1989). Different types of anaesthetic (general for GI surgery versus local for gluteal biopsy) were used in the two samples, and the amount present in the surgical biopsies would not have been as uniform as that present in the gluteal biopsies, a factor that may have influenced the results. Furthermore, in the surgical samples, the time at which the biopsy was taken during the operation may have affected the results. A recent paper shows that plasma ET levels increase gradually during surgery, and this phenomenon is dependent on the surgical procedure carried out (Shirakami *et al.*, 1995). Desensitisation following raised levels of ET-1 may have contributed to the depressed response to exogenous ET-1 during cooling, since the time of biopsy was not taken into account in the present study. However, it is unlikely that only the biopsies studied at 24°C, and not those at 37°C, were taken at the end of surgery when levels of ET were high.

In arteries from Raynaud's patients, cooling had no significant effect on responses to ET-1, either in intact or de-endothelialised vessels. These results indicate that, unlike control vessels, cooling modulates neither endothelial function or smooth muscle sensitivity to ET-1 in response to exogenous ET-1 in arteries from Raynaud's patients. At 37°C, there was a tendency for intact arteries from Raynaud's patients to have a higher sensitivity to ET-1 compared to denuded vessels, suggesting endothelium-derived constrictor factors are released. The presence of the endothelium did not appear to affect the response to ET-1 at 24°C. These results suggest that in Raynaud's patients the endothelium has a greater modulatory role at 37°C compared to 24°C. Perhaps then cooling causes the endothelium to malfunction, whereby it can no longer release constrictor substances. Alternatively, the generation of endothelium-derived dilator substances may be increased during cooling in arteries from Raynaud's patients, which would counteract the enhanced constriction (see Chapter 6).

When comparing responses in intact vessels obtained from patients with Raynaud's disease with those from healthy subjects, it was found that the sensitivity to ET-1 was higher in Raynaud's patients at 37°C. This difference probably arose from the combination of the release of endothelium-derived constrictors in arteries from Raynaud's patients, and dilator release in arteries from control subjects, as discussed above. Interestingly, at 24°C, no significant difference in responsiveness to ET-1 was found between Raynaud's patients and controls, in either intact or denuded arteries. If ET is implicated in the pathogenesis of Raynaud's disease, one would expect to find either an enhanced response to exogenous ET-1 compared to controls, due to increased smooth muscle sensitivity, or a depressed response, due to smooth muscle desensitisation through receptor down-regulation if endogenous ET levels are increased during cooling in Raynaud's patients. The fact that no significant difference was found would imply that ET is not a primary candidate in the pathogenesis of Raynaud's disease.

From the plasma samples taken from the volunteers, antinuclear antibodies (ANA) were found to be present in one control subject and four Raynaud's patients. The presence of such antibodies are associated with the development of connective tissue disease, and can be present many years before clinical symptoms arise. Only one of the volunteers presented with a connective tissue disease (Sjögren's syndrome), and was thus classified as having secondary Raynaud's phenomenon, but primary Raynaud's disease can precede the onset of rheumatological diseases by many years (Wollersheim *et al.*, 1989). It is therefore impossible to accurately classify subjects as either primary or secondary Raynaud's patients. No correlation was found between the presence of ANA and the response to ET-1 in the vessels obtained from patients with ANA compared to those without.

The sensitivity to the $\alpha_1$-adrenoceptor agonist, phenylephrine (PE), was increased during cooling in rat mesenteric resistance arteries with an intact endothelium. On initial inspection, these findings are in contrast to studies carried out in large arteries and veins, where cooling augments contractile responses to $\alpha_2$- but not $\alpha_1$-adrenergic agonists (Flavahan *et al.*, 1985). This may reflect differences between large vessels and resistance arteries, or differences in the type of blood vessel under study; acute cooling has been shown to decrease contraction to PE in the canine femoral vein (Flavahan & Vanhoutte, 1986), whilst having no significant effect on the response in canine saphenous veins (Flavahan *et al.*, 1985). However, Flavahan & Vanhoutte (1986) have shown a differential effect of cooling on the response to PE which is dependent on the length of time for which the vessels are cooled. Prolonged cooling - for 30 min - was found to potentiate the contractile response to PE in canine saphenous veins (Flavahan & Vanhoutte, 1986). Since the cooling time in the studies carried out in this thesis exceeded 30 min, the present results are in agreement with those of Flavahan & Vanhoutte (1986). The cold-induced increase in sensitivity to PE appeared to be partly mediated by an endothelium-derived vasoconstrictor in the present study, probably ET, since denudation or addition of the $ET_{A/B}$-receptor antagonist, bosentan,

reduced the increase. In agreement with these findings, Prasad *et al.* (1991) have shown that $\alpha_1$-adrenoceptors, but not $\alpha_2$-adrenoceptors, are responsible for mediating catecholamine-induced release of ET-1 from endothelial cells in culture. In addition, rapid release of ET-1 from cultured endothelial cells has been demonstrated, perhaps through *de novo* synthesis (Kurihara *et al.*, 1989b) or release from secretory stores (Milner *et al.*, 1990), on a time scale which would fit with the above proposal of $\alpha_1$-adrenoceptor-mediated stimulation of ET-1 activity. The present results, therefore, suggest that ET might contribute to the cold-induced potentiation of $\alpha$-adrenoceptor-mediated contraction.

The fact that denudation or the presence of bosentan had no effect on the response to PE at 37°C suggests that PE was not stimulating the release of endothelium-derived factors at this temperature. It would thus appear that simultaneous cooling and $\alpha_1$-adrenoceptor activation is required to induce the release of ET in rat mesenteric resistance arteries. At 24°C, bosentan caused a further rightward shift of the concentration-response curve to PE than removal of the endothelium. The most likely reason for this is that in the presence of the $ET_{A/B}$ receptor antagonist, endothelium-derived dilator substances were still able to oppose vasoconstriction, whereas denudation removed both constrictor and dilator substances.

Other evidence for the endothelium modulating the response to contractile agents during cooling comes from the studies using potassium chloride (KCl). In intact rat mesenteric arteries the contractile response was depressed at 24°C compared to that at 37°C. The depressant effect of cooling on the contractile response of KCl has been attributed to a decrease in calcium ion permeability of the vascular smooth muscle cell membrane (Vanhoutte, 1980). The present results, however, would suggest that this effect is at least partly mediated by the release of endothelium-derived dilator substances, probably NO and $PGI_2$, since removal of the endothelium or the presence of L-NAME/indomethacin abolished the reduction in maximal contraction during

cooling. The results from the experiments using L-NAME/indomethacin imply that EDHF is not involved in modulating the response to KCl during cooling.

In a similar study, cooling to 24°C was found to attenuate the response to KCl (50mM) in both rabbit ear and femoral arteries (Monge *et al.*, 1993). However, removal of the endothelium only augmented the contraction to KCl during cooling in ear arteries, and not in femoral arteries. This led Monge and colleagues (1993) to conclude that cooling possibly facilitates the release of endothelium-derived dilators only in the cutaneous vasculature. Clearly, the results from the present study do not agree with this finding. There is a possibility that mesenteric arteries, in some species at least, behave more like cutaneous vessels than deep, non-cutaneous vessels such as the femoral artery used in the study by Monge *et al.* (1993). Cooling has been shown to augment contraction induced by electrical field stimulation in canine cutaneous veins (Vanhoutte & Shepherd, 1970), and in mesenteric vessels (Rogers *et al.*, 1965; Vanhoutte & Lorenz, 1970), whilst depressing those responses in the femoral vein (Vanhoutte & Lorenz, 1970). Similar cooling-induced enhancement of sympathetic stimulation has been demonstrated in rat mesenteric arteries (Malik, 1969). Vanhoutte (1980) suggested that the mesenteric vasculature may resemble cutaneous vessels because it is exposed to local variations in temperature during food and water consumption.

# CHAPTER 5:
# AN INVESTIGATION OF VASOCONSTRICTOR SUBSTANCES IN VIVO

## 5.1. Introduction

At present, the physiological mechanisms underlying cold-induced vasoconstriction are poorly understood. Although there is some evidence for involvement of adrenoceptors, particularly $\alpha_2$-adrenoceptors, and of nitric oxide (NO), the majority of studies investigating the effects of temperature on vascular tone have been carried out using isolated blood vessels, and there has been little work in studies *in vivo*. There has been no work, as yet, exploring the role of endothelin (ET) in cold-induced vasoconstriction using specific receptor antagonists, even though there is evidence to suggest that ET-1 may play an important role in vasospasm. In the studies presented in this chapter, potential mediators of cold-induced vasoconstriction were examined in the autoperfused rat hindlimb model, using $\alpha$-adrenoceptor antagonists and ET-receptor antagonists.

## 5.2. Autoperfused hindlimb of the anaesthetised rat

### 5.2.1. The role of $\alpha$-adrenoceptors in mediating cold-induced vasoconstriction

The effects of the $\alpha$-adrenoceptor antagonists on the cold-induced rise in hindlimb perfusion pressure (HLPP) are shown in Figures 5.1 - 5.4. Basal HLPP (mmHg) and slopes of the temperature-HLPP curves before and after administration of the $\alpha$-adrenoceptor antagonists are shown in Table 5.1.

From Figure 5.1 and Table 5.1 it can be seen that the non-selective $\alpha_1/\alpha_2$ -adrenoceptor antagonist, phentolamine, significantly attenuated the rise in HLPP induced by cooling ($P < 0.05$). The presence of either the $\alpha_1$-adrenoceptor antagonist prazosin (Figure 5.2, Table 5.1), or the $\alpha_2$-adrenoceptor antagonist yohimbine (Figure 5.3, Table 5.1) alone, failed to produce such an attenuation ($P = 0.84$ and 0.54, for prazosin and yohimbine repectively). The combination of prazosin and yohimbine was unable to mimic the effect of phentolamine ($P = 0.30$), but was more

effective in reducing the slope of the temperature-HLPP curve than either antagonist given alone (Figure 5.4, Table 5.1).

From Table 5.1, it can be seen that prazosin and yohimbine significantly reduced the basal HLPP ($P < 0.01$ and 0.01, for prazosin and yohimbine repectively).

### 5.2.2. The role of endothelin in mediating cold-induced vasoconstriction

The effects of ET-receptor antagonists on the cold-induced rise in hindlimb perfusion pressure (HLPP) are shown in Figures 5.5 and 5.6. Basal HLPP (mmHg) and slopes of the temperature-HLPP curves before and after administration of the ET-receptor antagonists are shown in Table 5.2.

From Figure 5.5 and Table 5.2 it can be seen that the non-selective $ET_{A/B}$-receptor antagonist, bosentan, had no effect on the rise in HLPP induced by cooling ($P = 0.29$). The presence of the selective $ET_A$-receptor antagonist BQ-123 attenuated the rise, but this failed to reach significance ($P = 0.09$) (Figure 5.6, Table 5.2).

| Drug | Basal HLPP | Slope |
|---|---|---|
| Control | 92 ± 2 | 16.72 ± 1.67 |
| Phentolamine | 83 ± 8 | 10.07 ± 1.22 * |
| Control | 120 ± 6 | 17.14 ± 1.48 |
| Prazosin | 103 ± 5 ** | 17.38 ± 1.22 |
| Control | 110 ± 2 | 16.63 ± 1.26 |
| Yohimbine | 93 ± 4 ** | 15.89 ± 0.30 |
| Control | 119 ± 10 | 17.76 ± 2.25 |
| Prazosin & Yohimbine | 98 ± 7 | 14.65 ± 0.97 |

***Table 5.1.*** *Basal HLPP (mmHg) at 37°C and slope of temperature-HLPP curves before (control) and after antagonist: phentolamine, 10μg/kg; prazosin, 100μg/kg; yohimbine, 300μg/kg. All values are mean ± SEM. n=4-6 animals in each group. *P<0.05, **P<0.01 with respect to control; paired t-test.*

| Drug | Basal HLPP | Slope |
|---|---|---|
| Control | 136 ± 10 | 18.32 ± 2.75 |
| Bosentan | 130 ± 12 | 21.50 ± 3.39 |
| Control | 114 ± 9 | 18.51 ± 1.66 |
| BQ-123 | 111 ± 7 | 13.41 ± 3.48 |

***Table 5.2.*** *Basal HLPP (mmHg) at 37°C and slope of temperature-HLPP curves before (control) and after antagonist: bosentan, 1000μg/kg; BQ-123, 1000μg/kg. All values are mean ± SEM. n=5 and 6 animals for bosentan and BQ-123 group respectively.*

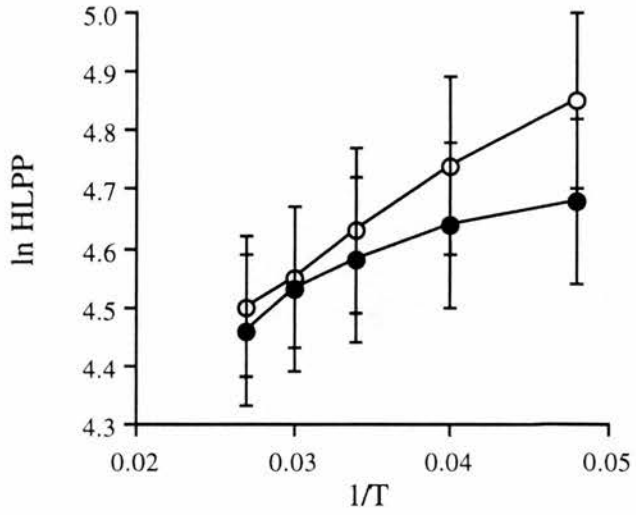



**Figure 5.1.** *The effect of phentolamine on the cold-induced rise in hindlimb perfusion pressure (HLPP): temperature (T)-HLPP curves from 37°C to 21°C; control (○) and in the presence of phentolamine (10μg/kg) (●). n=5 animals.*

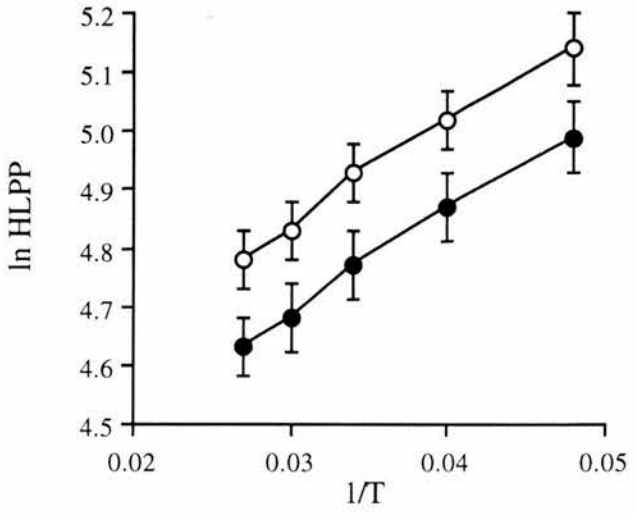



***Figure 5.2.*** *The effect of prazosin on the cold-induced rise in hindlimb perfusion pressure (HLPP): temperature (T)-HLPP curves from 37°C to 21°C; control (○) and in the presence of prazosin (100μg/kg) (●). n=6 animals.*

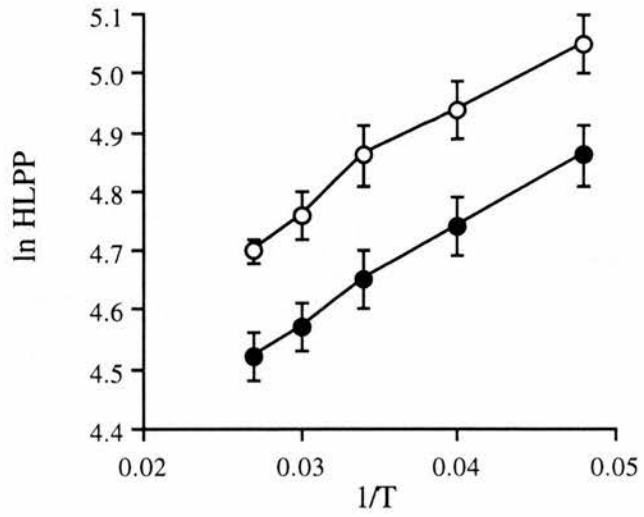



***Figure 5.3.*** *The effect of yohimbine on the cold-induced rise in hindlimb perfusion pressure (HLPP): temperature (T)-HLPP curves from 37°C to 21°C; control (○) and in the presence of yohimbine (300μg/kg) (●). n=6 animals.*

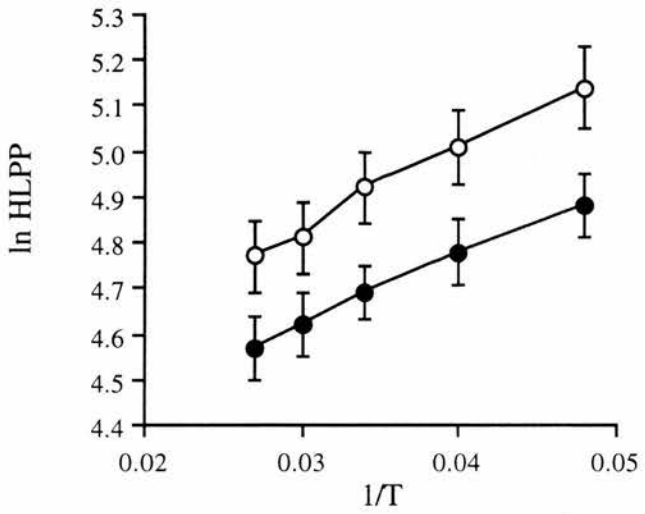



***Figure 5.4.*** *The effect of combined prazosin and yohimbine on the cold-induced rise in hindlimb perfusion pressure (HLPP): temperature (T)-HLPP curves from 37°C to 21°C; control (○) and in the presence of prazosin (100μg/kg) and yohimbine (300μg/kg) (●). n=4 animals.*

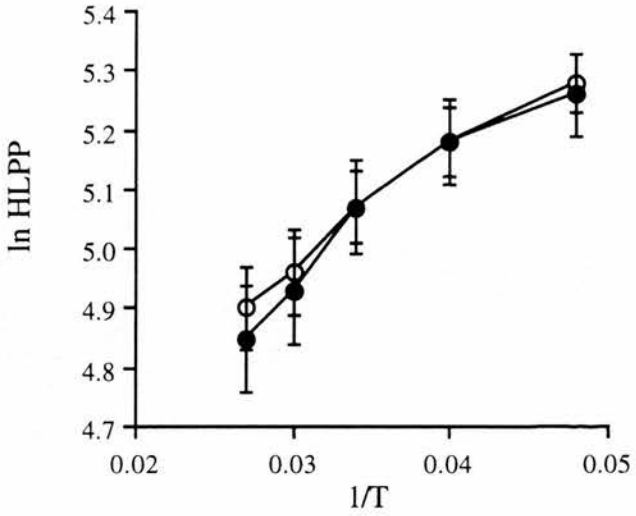



***Figure 5.5.*** *The effect of bosentan on the cold-induced rise in hindlimb perfusion pressure (HLPP): temperature (T)-HLPP curves from 37°C to 21°C; control (○) and in the presence of bosentan (1000μg/kg) (●). n=5 animals.*

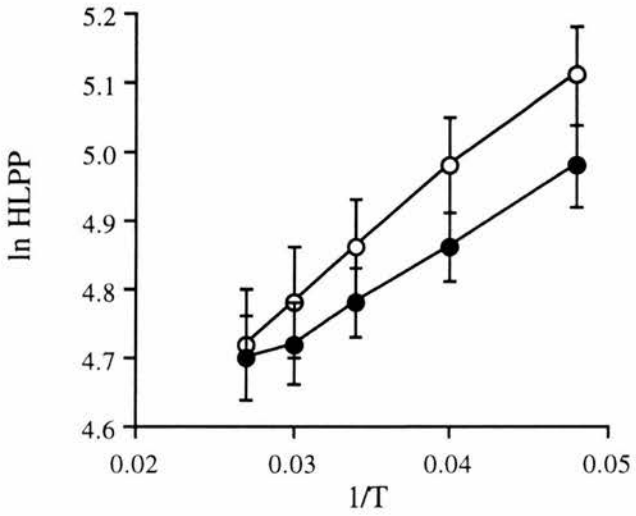



***Figure 5.6.*** *The effect of BQ-123 on the cold-induced rise in hindlimb perfusion pressure (HLPP): temperature (T)-HLPP curves from 37°C to 21°C; control (○) and in the presence of BQ-123 (1000μg/kg) (●). n=6 animals.*

## 5.3. Discussion

The effect of phentolamine suggested a role for α-adrenoceptors in mediating the cold-induced vasoconstriction in the rat hindlimb. However, the selective $\alpha_1$-antagonist, prazosin, and the selective $\alpha_2$-antagonist, yohimbine, failed to attenuate the cold-induced vasoconstriction. From the literature, $\alpha_2$-adrenoceptor-mediated vasoconstriction has been shown to be augmented by cooling (Flavahan *et al.*, 1985; Ekenvall *et al.*, 1988). One may therefore expect $\alpha_2$-adrenoceptors to be partly responsible for the cold-induced vasoconstriction in the present hindlimb model. Perhaps the lack of effect of yohimbine was due to an enhanced $\alpha_1$-adrenoceptor-mediated constriction, resulting from the blockade of $\alpha_2$-adrenoceptors located prejunctionally on nerve terminals (Illes & Norenberg, 1987) and on endothelial cells (Angus *et al.*, 1986), which inhibit noradrenaline release and mediate the release of endothelium-derived dilator substances, respectively. Thus, to eliminate the possibility that a simultaneous blockade of both α-adrenoceptors is required to mimic the effect of phentolamine, the effect of combining prazosin and yohimbine was studied. This combination was, however, unable to reproduce the effect of phentolamine, indicating that the action of phentolamine may not involve α-adrenoceptors. Phentolamine has also been reported to block ATP-sensitive $K^+$-channels (McPherson & Angus, 1989; Schwietert *et al.*, 1992), but this action would seem unlikely in the present study, since closure of such channels would favour increased vasoconstriction, which is the opposite of the effect it produced in the hindlimb. Another reported action of phentolamine is antagonism at 5-hydroxytrptamine (5-HT) receptors (Carroll *et al.*, 1977; Limberger *et al.*, 1989). Since 5-HT has been implicated in cold-induced vasoconstriction (Seibold, 1985) it is possible that the present results using phentolamine were due to blockade of 5-$HT_2$ receptors. This could be investigated by performing similar experiments in the presence of the 5-$HT_2$ receptor antagonist ketanserin.

It is perhaps worth noting that the basal HLPP was significantly reduced in the presence of prasozin and yohimbine, and had a tendency to be reduced when prasozin and yohimbine were given in combination. To eliminate the possibility that the level of basal vascular tone had influenced the effectiveness of the selective $\alpha_1$- and $\alpha_2$-adrenoceptor antagonists, the studies could be repeated in the presence of a vasoconstrictor which does not act on adrenoceptors, such as the thromboxane $A_2$ mimetic U46619, which would counteract any fall in HLPP induced by prazosin or yohimbine.

There would appear to be a role for endothelin (ET) in mediating cold-induced vasoconstriction, perhaps through the $ET_A$ receptor, because there was a tendency for the selective $ET_A$ receptor antagonist, BQ-123, to reduce the rise in hindlimb perfusion pressure during cooling, although this did not quite achieve statistical significance ($P$=0.09). Bolus administration of ET-1 has been shown to produce a marked increase in hindquarters blood flow in the rat (Gardiner *et al.*, 1989; Wright & Fozard, 1988). During the pressor phase, or during intravenous infusion where there was no initial fall in blood pressure, no change in hindquarters blood flow occurred, although there were marked decreases in blood flow to other vascular beds. Thus, ET may be more effective in activating vasodilator mechanisms in the hindquarter bed, perhaps due to a higher density of dilator $ET_B$ receptors present on endothelial cells or a reduced number of constrictor vascular smooth muscle $ET_{A/B}$ receptors, compared to other vascular beds. This may explain why the effect of BQ-123 failed to reach significance in the present study; the hindlimb might not be a sensitive model for investigating $ET_A$ receptors. Also, studies using the endothelin-converting enzyme (ECE) inhibitor, phosphoramidon, led Gardiner *et al.* (1991) to suggest that ECE activity may be lower in the rat hindquarters compared to other vascular beds. Hence, any cold-induced stimulation of ET production may not be as apparent in the present model as it might be in other vascular beds. An additional confounding factor is the presence of heparin in the circulating blood, which is administered in the hindlimb model to prevent

coagulation. Heparin has been shown to inhibit ET-1 generation in cultured endothelial cells, probably through the action of nitric oxide (NO) (Yokokawa *et al.*, 1993). For this reason, the experiments using BQ-123 could be repeated in the absence of heparin.

Bosentan, the non-selective $ET_{A/B}$-receptor antagonist, had no effect on cold-induced vasoconstriction. This probably reflects the additive effect of blocking smooth muscle $ET_A$ receptors, which would favour a decrease in cold-induced vasoconstriction as seen with BQ-123, and blocking endothelial $ET_B$ receptors, which would favour an increase in cold-induced vasoconstriction.

Further studies, using selective inhibitors of the nitric oxide and prostacyclin pathways, could be performed in order to investigate the role of endothelium-derived vasodilators in cold-induced vasoconstriction *in vivo*; if dilator function is increased during cooling, one would expect to find an enhanced vasoconstriction induced by cooling in the presence of such inhibitors.

# CHAPTER 6:
# AN INVESTIGATION OF VASODILATOR SUBSTANCES IN VITRO

## 6.1. Introduction

Several studies involving the use of blood vessels from animals have revealed that cooling can potentiate the release and/or response to nitric oxide (NO). In experiments using rat aortic strips, Karaki & Nagase (1987) demonstrated that lowering the bath temperature from 37°C to 33°C increased relaxation to the endothelium-dependent and -independent vasodilators, carbachol and sodium nitroprusside (SNP), respectively. Since the response to both dilators was similarly enhanced, it appears that the vascular smooth muscle sensitivity to NO is augmented during cooling. Monge *et al.* (1991) examined the effect of $N^G$-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO synthase, and also de-endothelialisation on the responses to endothelin-1 (ET-1) at 37°C and 24°C. Their findings suggest that the decrease in sensitivity to ET-1 found during cooling is caused either by an increased availability of NO, which could be due to increased generation or reduced clearance or, alternatively, by increased sensitivity to NO.

If this increase in NO-mediated responses during cooling is absent in the vascular endothelium of patients with Raynaud's disease, possibly through injury to the endothelial cells, increased production and enhanced constriction to ET-1 could cause vasospasm. Evidence that the endothelium may be injured in Raynaud's disease comes from studies showing that factor VIII and von Willebrand factor antigen levels are raised in Raynaud's patients (Kahaleh *et al.*, 1981), and higher levels are associated with greater severity of the disease (Lau *et al.*, 1991).

The studies presented in this chapter examined the relaxant responses to the endothelium-dependent vasodilator, acetylcholine (ACh), and the endothelium-independent vasodilator, SNP, in resistance arteries obtained from (i) rat mesentery and (ii) gluteal fat biopsies taken from control subjects and patients with Raynaud's disease. It has been shown that the response to exogenous nitrovasodilators is potentiated when endogenous endothelium-dependent dilatation is depressed, possibly

due to hypersensitivity of the soluble guanylate cyclase enzyme in smooth muscle cells (Shirasaki & Su, 1985; Moncada *et al.*, 1991). In order to prevent such a modulating effect of the endothelium in the present study, vessels were de-endothelialised prior to generating concentration-relaxation curves to SNP. Any effect of temperature on the response to SNP would therefore be the result of a change in smooth muscle sensitivity to NO. In addition, the effect of cooling on the vasorelaxation to the endothelium-dependent and -independent vasodilators was investigated. This is an important factor which, until now, has not been addressed in patients with Raynaud's disease.

## 6.2. Small vessel arteriograph (perfusion myograph)

### 6.2.1. The effect of cooling on the relaxation to acetylcholine

#### 6.2.1.1. Rat mesenteric resistance arteries

Mean resting lumen diameter, % contraction to phenylephrine (PE) and % relaxation to ACh did not differ significantly between the group at 37°C and the group at 24°C (see Table 6.1).

**TABLE 6.1.** *Baseline data for vessels studied at 37°C and 24°C*

| | 37°C | 24°C |
|---|---|---|
| LD (μm) | 331 ± 10 | 308 ± 13 |
| % Contraction to PE | 73 ± 1 | 71 ± 2 |
| % Relaxation to ACh | 100 ± 0 | 95 ± 2 |

*LD, resting lumen diameter; PE, phenylephrine ($10^{-5}$M); ACh, acetylcholine ($10^{-6}$M). Values are mean ± SEM for n = 6 vessels at each temperature (total n = 12/12).*

Cooling to 24°C caused a small rightward shift of the concentration-relaxation curve to ACh (Figure 6.1), but this failed to reach statistical significance ($EC_{50}$ = 1.2 ± 0.3 x $10^{-7}$M at 37°C vs. 1.8 ± 0.6 x $10^{-7}$M at 24°C; $P$=0.36). The $E_{max}$ for ACh at 24°C was not significantly different from that at 37°C (101 ± 2 at 37°C vs. 96 ± 2 at 24°C; $P$=0.09).

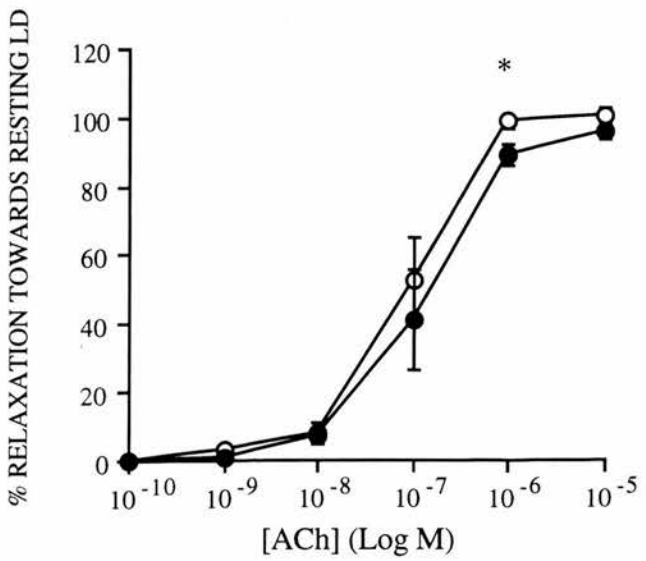



***Figure 6.1.*** *The effect of cooling on the relaxant response to ACh in rat mesenteric resistance arteries preconstricted with PE ($10^{-5}$M): concentration-relaxation curves to ACh (expressed as % relaxation towards resting lumen diameter (LD)) at 37°C (○; n=6) and at 24°C (●; n=6). All values are mean ± SEM. *P < 0.05 (unpaired t-test).*

### 6.2.1.2. Human resistance arteries from gluteal biopsies

Mean resting lumen diameter, % contraction to KCl and % relaxation to ACh did not differ significantly between the control subject group, at 37°C and 24°C, and the Raynaud's patient group at 37°C and 24°C (see Table 4.11, Chapter 4).

In arteries obtained from control subjects, there was no significant effect of cooling on the concentration-relaxation curve to ACh, although there was a trend of a rightward-shift and a decrease in the maximal relaxation (Figure 6.2a), ($EC_{50}$ = 7.8 ± 2.8 x $10^{-8}$M at 37°C vs. 1.4 ± 0.4 x $10^{-7}$M at 24°C; $P$=0.29. $E_{max}$ = 88 ± 4 at 37°C and 74 ± 9 at 24°C; $P$=0.21). (Tables 6.2 & 6.3). Figure 6.2b shows the effect of cooling on the relaxant response to ACh in vessels from Raynaud's patients. There was no significant change in sensitivity to ACh during cooling ($EC_{50}$ = 8.4 ± 5.6 x $10^{-8}$M at 37°C vs. 1.7 ± 0.9 x $10^{-7}$M at 24°C; $P$=0.42) (Table 6.2), but there was a marked enhancement in the maximal relaxation attainable at 24°C ($E_{max}$ = 44 ± 14 at 37°C and 86 ± 3 at 24°C; $P$=0.01) (Table 6.3).

When comparing the relaxant responses to ACh between arteries from control subjects and those from Raynaud's patients, the maximal relaxation was found to be significantly depressed in Raynaud's patients at 37°C ($E_{max}$ = 88 ± 4 for controls vs. 44 ± 14 for Raynaud's patients; $P$<0.01) (Table 6.3) (Figure 6.3a). There was no significant difference in sensitivity to ACh between the groups ($EC_{50}$ = 7.8 ± 2.8 x $10^{-8}$M for controls vs. 8.4 ± 5.6 x $10^{-8}$M for Raynaud's patients; $P$=0.93) (Table 6.2), although there was a rightward-shift of the concentration-relaxation curve in the Raynaud's group (Figure 6.3a). Interestingly, at 24°C the sensitivity and maximal relaxation to ACh were not significantly different between controls and Raynaud's patients; indeed, there was a tendency for the relaxation in vessels from Raynaud's patients to be greater than that found in vessels from control subjects ($EC_{50}$ = 1.4 ± 0.4 x $10^{-7}$M for controls vs. 1.7 ± 0.9 x $10^{-7}$M for Raynaud's patients; $P$=0.73. $E_{max}$ = 74 ± 9 for controls vs. 86 ± 3 for Raynaud's patients; $P$=0.24) (Tables 6.2 & 6.3) (Figure 6.3b).

***TABLE 6.2.*** *$EC_{50}$ values for ACh concentration-relaxation curves in human resistance arteries obtained from gluteal biopsies*

| | Control | Raynaud's |
|---|---|---|
| 37°C | $7.8 \pm 2.8 \times 10^{-8}$M | $8.4 \pm 5.6 \times 10^{-8}$M |
| 24°C | $1.4 \pm 0.4 \times 10^{-7}$M | $1.7 \pm 0.9 \times 10^{-7}$M |

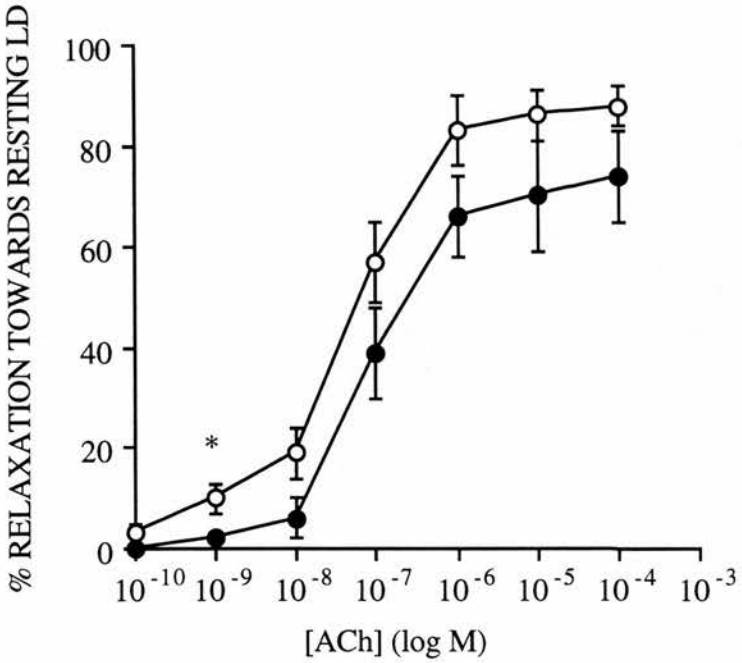
*Values are mean ± SEM for n = 6 vessels in each group.*

***TABLE 6.3.*** *$E_{max}$ values for ACh concentration-relaxation curves in human resistance arteries obtained from gluteal biopsies*

| | Control | Raynaud's |
|---|---|---|
| 37°C | 88 ± 4 | 44 ± 14 †† |
| 24°C | 74 ± 9 | 86 ± 3 * |

*Values are mean ± SEM for n = 6 vessels in each group. *P < 0.05 compared to 37°C; ††P < 0.05 compared to control group (unpaired t-test).*

**a**




**b**

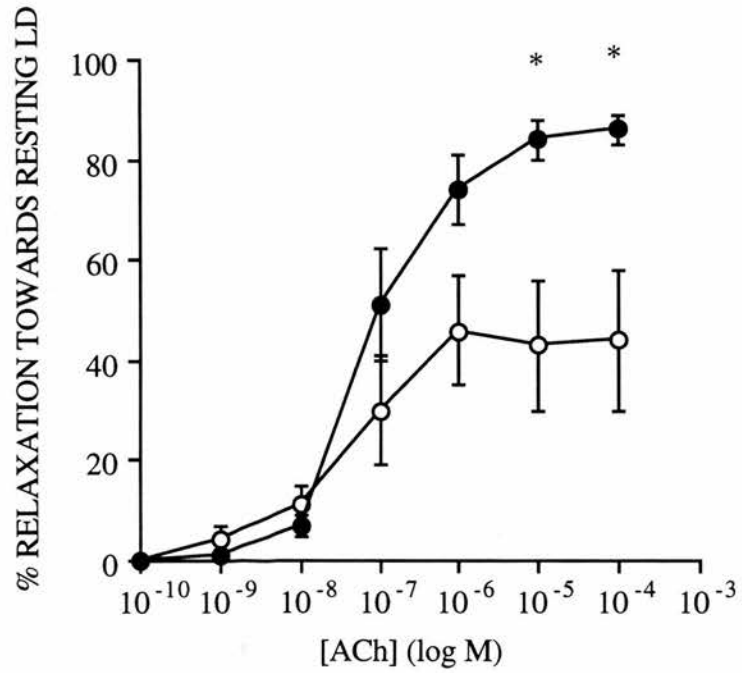
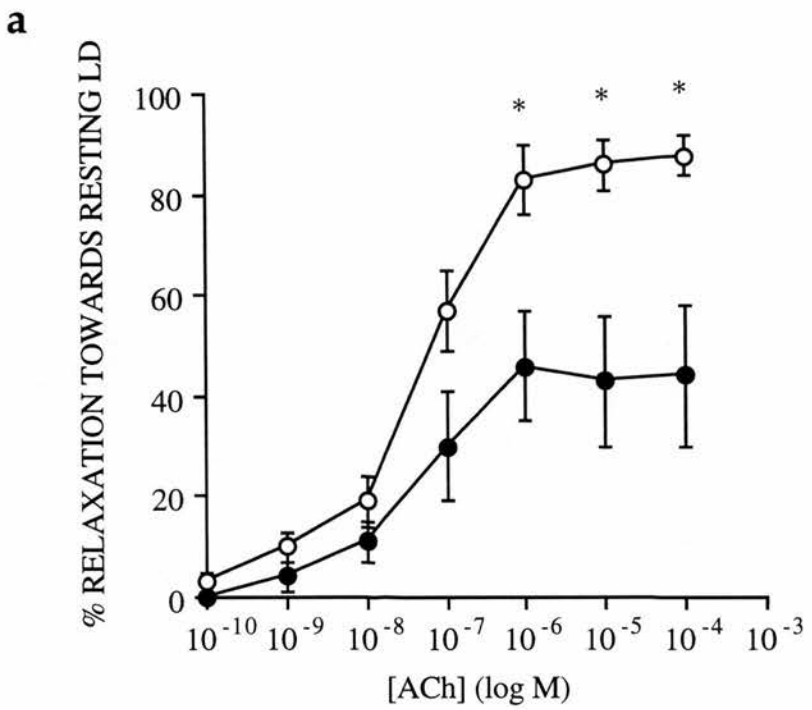



***Figure 6.2.*** *The effect of cooling on the relaxant response to ACh in human subcutaneous resistance arteries preconstricted with NA ($10^{-5}$M): concentration-relaxation curves to ACh (expressed as % relaxation towards resting lumen diameter (LD)) in vessels from control subjects (**Figure 6.2a**) and Raynaud's patients (**Figure 6.2b**) at 37°C (○; n=6) and at 24°C (●; n=6). All values are mean ± SEM. *P < 0.05 (unpaired t-test; ANOVA).*



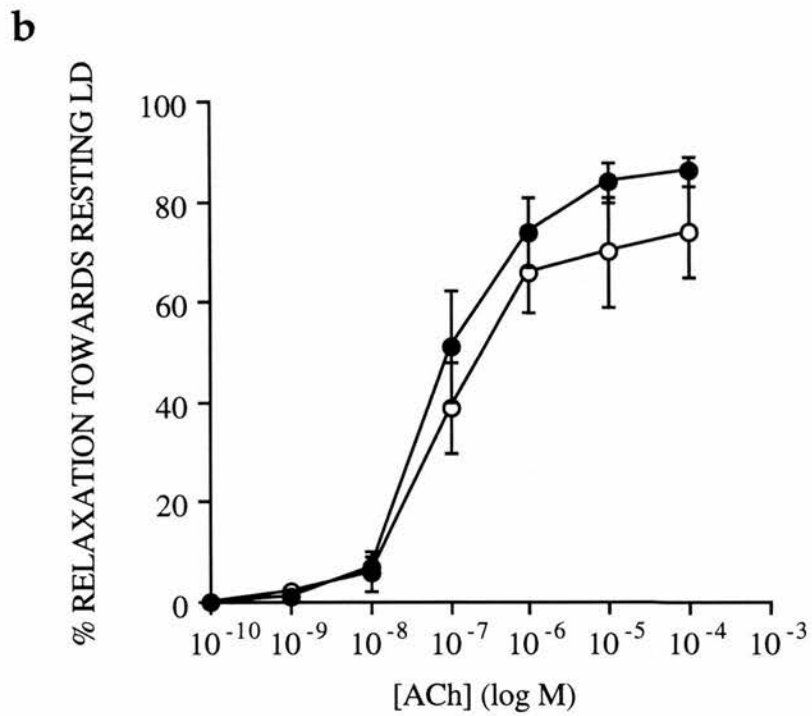




***Figure 6.3.*** *A comparison between the relaxant response to ACh in subcutaneous resistance arteries isolated from Raynaud's patients and control subjects preconstricted with NA ($10^{-5}$M): concentration-relaxation curves to ACh (expressed as % relaxation towards resting lumen diameter (LD)) at 37°C (**Figure 6.3a**) and at 24°C (**Figure 6.3b**) in vessels from control subjects (○; n=6) and Raynaud's patients (●; n=6). All values are mean ± SEM. *P<0.05 (unpaired t-test; ANOVA).*

### 6.2.2. The effect of cooling on relaxation induced by sodium nitroprusside

#### 6.2.2.1. Rat mesenteric resistance arteries

Mean resting lumen diameter and % contraction to PE did not differ significantly between the group at 37°C and the group at 24°C, but there was a significant difference in % relaxation to ACh between the groups (Table 6.4).

***TABLE 6.4.*** *Baseline data for vessels studied at 37°C and 24°C*

| | 37°C | 24°C |
|---|---|---|
| LD (μm) | 323 ± 10 | 311 ± 14 |
| % Contraction to PE | 73 ± 1 | 72 ± 1 |
| % Relaxation to ACh | 100 ± 0 | 93 ± 2 * |

*LD, resting lumen diameter; PE, phenylephrine ($10^{-5}$M); ACh, acetylcholine ($10^{-6}$M). Values are mean ± SEM for n = 6 vessels at each temperature (total n = 12/12). *P < 0.05 (unpaired t-test).*

Cooling to 24°C tended to cause a small rightward shift of the concentration-relaxation curve to SNP (Figure 6.4), but this did not reach statistical significance ($EC_{50}$ = 6.2 ± 3.9 x $10^{-8}$M at 37°C vs. 6.2 ±1.4 x $10^{-8}$M at 24°C; $P$=0.99). The $E_{max}$ for SNP at 24°C was not significantly different to that at 37°C (93 ± 2 at 37°C vs. 88 ± 4 at 24°C; $P$=0.36).

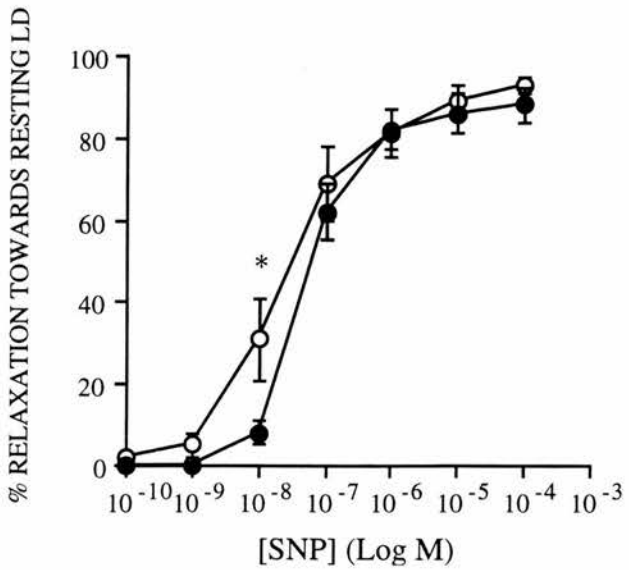



***Figure 6.4.*** *The effect of cooling on the relaxant response to SNP in rat mesenteric resistance arteries preconstricted with PE ($10^{-5}$M): concentration-response curves to SNP (expressed as % relaxation towards resting lumen diameter (LD)) at 37°C (○; n=6) and at 24°C (●; n=6). All values are mean ± SEM. *P < 0.05 (unpaired t-test).*

### 6.2.2.2. Human subcutaneous resistance arteries from gluteal biopsies

Mean resting lumen diameter, % contraction to KCl and % relaxation to ACh did not differ significantly between the control subject group, at 37°C and 24°C, and the Raynaud's patient group, at 37°C and 24°C (see Table 4.11, Chapter 4).

In vessels from control subjects, cooling decreased the sensitivity to SNP, as seen by a rightward shift of the concentration-relaxation curve ($EC_{50}$ = 1.8 ± 0.5 x $10^{-7}$M at 37°C vs. 2.1 ± 0.7 x $10^{-6}$M at 24°C; *P*=0.01) (Table 6.5) (Figure 6.5a). The difference in maximal relaxation was not statistically significant ($E_{max}$ = 92 ± 15 at 37°C vs. 80 ± 5 at 24°C; *P*=0.45) (Table 6.6). Cooling did not significantly reduce sensitivity or maximal relaxation to SNP in arteries obtained from Raynaud's patients, but there was a tendency for both to be reduced at 24°C ($EC_{50}$ = 3.0 ± 1.8 x $10^{-6}$M at 37°C vs. 1.6 ± 1.1 x $10^{-5}$M at 24°C; *P*=0.29. $E_{max}$ = 73 ± 9 at 37°C vs. 67 ± 9 at 24°C; *P*=0.65) (Tables 6.5 & 6.6) (Figure 6.5b).

Although there were no significant differences in the relaxation to SNP between arteries from Raynaud's patients and those from controls, at 37°C the concentration-relaxation curve generated in Raynaud's vessels lay to the right of the curve from control subjects ($EC_{50}$ = 1.8 ± 0.5 x $10^{-7}$M for controls vs. 3.0 ± 1.8 x $10^{-6}$M for Raynaud's patients; *P*=0.14) (Table 6.5) (Figure 6.6a). Also, there was a tendency for the maximal relaxation to SNP to be reduced in vessels from Raynaud's patients compared to that in controls ($E_{max}$ = 92 ± 15 for controls vs. 73 ± 9 for Raynaud's patients; *P*=0.30 at 37°C.$E_{max}$ = 80 ± 5 for controls vs. 67 ± 9 for Raynaud's patients; *P*=0.28 at 24°C) (Table 6.6) (Figures 6.6a and 6.6b).

***TABLE 6.5.*** *$EC_{50}$ values for SNP concentration-relaxation curves in human resistance arteries obtained from gluteal biopsies*

| | Control | Raynaud's |
|---|---|---|
| 37°C | $1.8 \pm 0.5 \times 10^{-7}$M | $3.0 \pm 1.8 \times 10^{-6}$M |
| 24°C | $2.1 \pm 0.7 \times 10^{-6}$M * | $1.6 \pm 1.1 \times 10^{-5}$M |

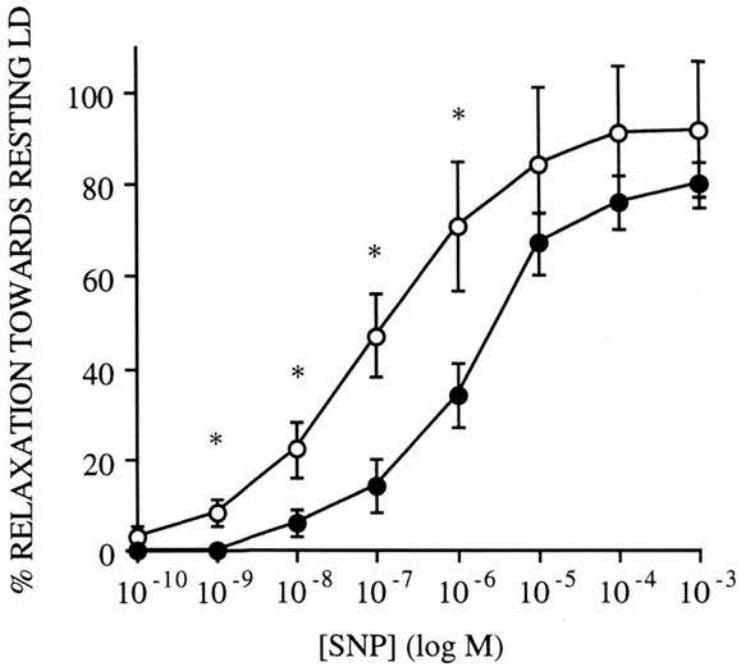
*Values are mean ± SEM for n = 6 vessels in each group. *P < 0.05 compared to 37°C (unpaired t-test).*

***TABLE 6.6.*** *$E_{max}$ values for SNP concentration-relaxation curves in human resistance arteries obtained from gluteal biopsies*

| | Control | Raynaud's |
|---|---|---|
| 37°C | 92 ± 15 | 73 ± 9 |
| 24°C | 80 ± 5 | 67 ± 9 |

*Values are mean ± SEM for n = 6 vessels in each group.*

**a**




**b**

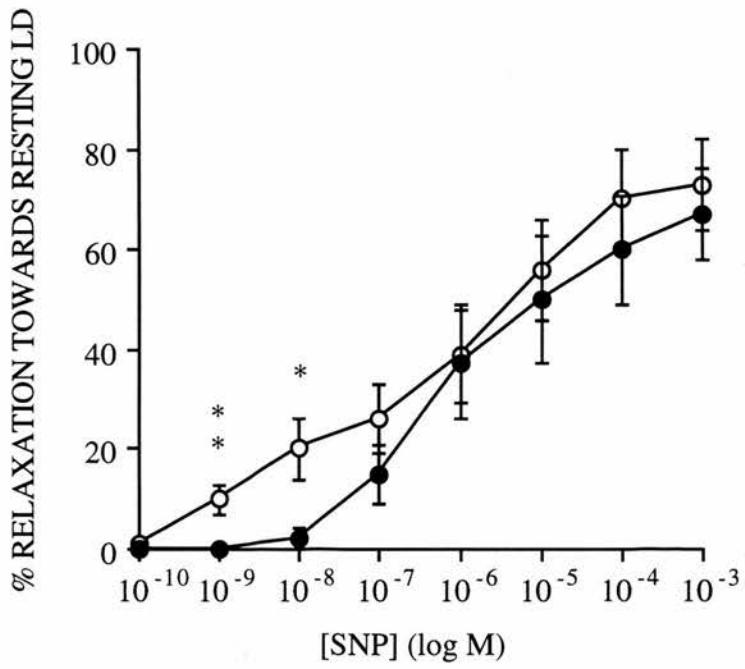
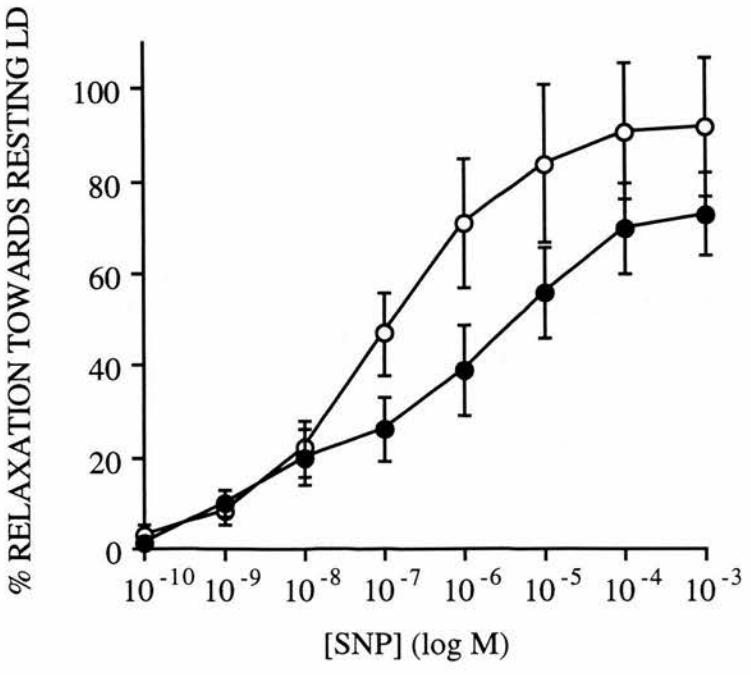



***Figure 6.5.*** *The effect of cooling on the relaxant response to SNP in human subcutaneous resistance arteries preconstricted with NA ($10^{-5}$M): concentration-relaxation curves to SNP (expressed as % relaxation towards resting lumen diameter (LD)) in vessels from control subjects (**Figure 6.5a**) and Raynaud's patients (**Figure 6.5b**) at 37°C (○; n=6) and at 24°C (●; n=6). All values are mean ± SEM. *P < 0.05, **P < 0.01 (unpaired t-test; ANOVA).*

**a**

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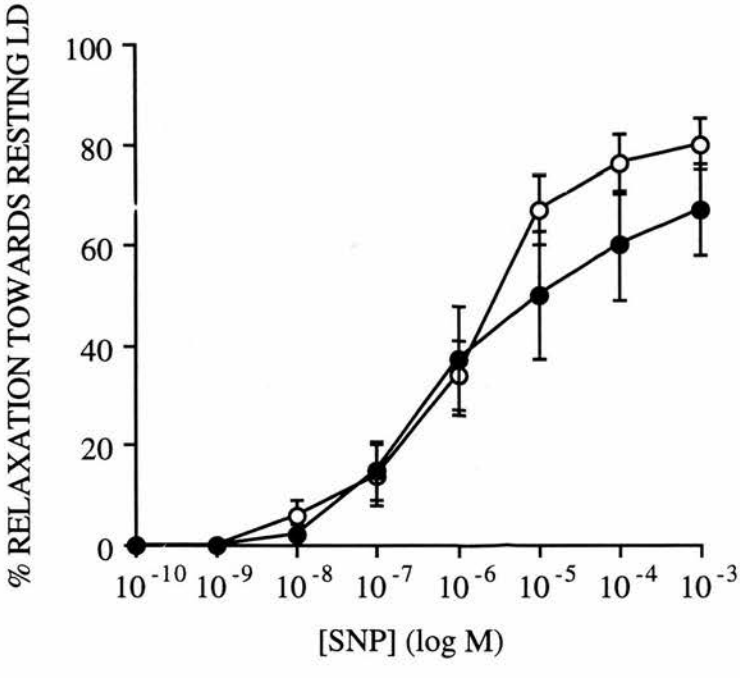


***Figure 6.6.*** *A comparison between the relaxant response to SNP in subcutaneous resistance arteries isolated from Raynaud's patients and control subjects preconstricted with NA ($10^{-5}$M): concentration-relaxation curves to SNP (expressed as % relaxation towards resting lumen diameter (LD)) at 37°C (**Figure 6.6a**) and at 24°C (**Figure 6.6b**) in vessels from control subjects (○; n=6) and Raynaud's patients (●; n=6). All values are mean ± SEM.*

### 6.1.4. Discussion

The results from the studies in rat mesenteric resistance arteries showed there was no significant effect of cooling on the dilator response to the endothelium-dependent vasodilator, acetylcholine (ACh). However, a more marked effect was seen using subcutaneous vessels taken from control subjects, in which cooling appeared to depress the activity of endothelium-derived dilator substances. Perhaps the difference between the human and rat data reflect species differences or differences between cutaneous arteries and deep arteries; because of the constant changes in their physiological environment, cutaneous vessels may adapt in order to function optimally during changes in temperature. Responses to temperature changes may therefore be more pronounced in cutaneous vessels than in deep vessels. Evidence to support such an adaptation in the cutaneous vasculature comes from a study where the potentiating effect of cooling on the responsiveness of rabbit ear arteries was shown to be more pronounced in animals which were housed at 5°C, than those kept at 20°C (McClelland *et al.*, 1969).

The fact that cooling tended to attenuate the relaxant response to ACh in control subjects implies that there may be inhibition at some point in the production and/or release of nitric oxide (NO), prostacyclin ($PGI_2$) and/or endothelium-derived hyperpolarising factor (EDHF) at 24°C. Studies using fetal cotyledons from sheep have shown that prostaglandin H synthase (PGHS) activity is maximal near the normal body temperature of sheep - 39°C - and cooling markedly reduces enzyme activity (Wimsatt *et al.*, 1995). Since prostaglandin $H_2$ ($PGH_2$) is the common precursor of the prostanoids, these findings provide support for a cold-induced reduction in $PGI_2$ production. There is, to date, no information available regarding the effect of temperature on NO synthase (NOS) activity.

The fact that the relaxant response to SNP in arteries from control subjects was attenuated during cooling suggests that the responsiveness of the vascular smooth

muscle to NO may itself be reduced. Possible mechanisms for a cold-induced decrease in smooth muscle sensitivity to NO are: (1) a reduction in activity of soluble guanylate cyclase (sGC); (2) a decrease in cGMP levels via an enhanced activity of cGMP phosphodiesterase; or (3) a reduced sensitivity of the cGMP-dependent mechanisms responsible for relaxation. However, a recent study demonstrated that plasma levels of cGMP increased during cold exposure in healthy female controls but not in women with Raynaud's disease (Leppert *et al.*, 1995), implying that sGC activity is not depressed during cooling in control subjects. The second possibility would appear unlikely because cyclic nucleotide phosphodiesterases have been found to be insensitive to temperature (Amer & Kreighbaum, 1975). To further investigate the third possibility, studies could be carried out using 8-bromo cGMP, a membrane-permeable cGMP derivative, to activate cGMP-dependent protein kinases directly. A confounding factor in the observation that cooling depresses the response to SNP in control arteries is the ability of SNP to generate NO varies depending on the experimental conditions; cooling has been shown to decrease the amount of NO released from SNP (Feelisch & Noack, 1987). It is therefore difficult to determine whether the depressed response to SNP at 24°C was due to a change in smooth muscle sensitivity to NO or to a reduction in the release of NO.

The relaxant response to SNP in rat mesenteric resistance arteries was not significantly attenuated at 24°C compared to 37°C, but there was a trend for reduced dilatation during cooling. Perhaps statistical significance was unable to be achieved because the number of vessels studied was too low. Also, the group at 24°C displayed less relaxation to ACh before endothelial removal; this may have given rise to a potentiation of endothelium-independent dilatation (Moncada *et al.*, 1991), which would oppose any rightward-shift of the concentration-relaxation curve induced by cooling. In addition, as mentioned earlier, the difference between the rat and human results may reflect an adaptation to cooling in the cutaneous vasculature, although species differences cannot be excluded.

In arteries obtained from Raynaud's patients, ACh-induced vasodilatation was found to be significantly enhanced at 24°C compared to the response at 37°C. The fact that the relaxation to SNP was similar at both temperatures suggests that smooth muscle sensitivity to NO is not temperature-dependent in Raynaud's patients. This implies that the production and/or release of endothelium-dependent dilator substances are enhanced in arteries from Raynaud's patients at 24°C, or alternatively, that they are depressed at 37°C.

When comparing the relaxant responses between arteries from control subjects and Raynaud's patients it was found that endothelium-dependent relaxation was impaired at 37°C in Raynaud's vessels, implying that there is a dysfunction of the endothelium in Raynaud's disease. Unexpectedly however, no such impairment was found during cooling; the response to ACh at 24°C was similar to that found in vessels from control subjects. This suggests, therefore, that endothelium-dependent dilator activity is depressed at 37°C, but is normal at 24°C. Possible mechanisms for a decrease in dilator release include: (1) reduced activities of NO synthase (NOS) and/or $PGI_2$ synthase; (2) increased levels of endogenous NOS inhibitors, such as asymmetric dimethyl-L-arginine (ADMA) and $N^G$-monomethyl-L-arginine (L-NMMA); (3) reduced diffusibility of NO through altered permeability of cell membranes; (4) and augmented breakdown of NO by increased levels of superoxide anions. Each of these possibilities could be investigated as follows: (1) using radioimmunoassays to measure the enzymatic conversion of radiolabelled substrates in cultured endothelial cells; (2) measuring plasma levels of ADMA and L-NMMA by radioimmunoassay; (3) by exposing one side of a chamber divided by a lipid bilayer to NO and measuring the concentration of gas that has diffused through the membrane to the other side; and (4) carrying out experiments in the presence of the superoxide anion scavenger, superoxide dismutase (SOD). Perhaps during cooling there is a temperature-dependent improvement in the above mechanisms because no impairment of the response to ACh was found at 24°C. In the context of cold-induced vasospasm, however, this would

not appear to make sense. In the present findings it should be remembered that it was the agonist-stimulated generation of endothelium-dependent vasodilators that was examined, which need not necessarily reflect basal production *in vivo*. It is possible that during cooling production of endogenous NO is reduced to such a degree that there is up-regulation of the stimulated NO pathway. Because the responses to SNP were not similarly potentiated, it would appear that the up-regulation occurs at a site proximal to smooth muscle. NO has been shown to inhibit its own synthesis through a negative feedback mechanism on NOS (Assreuy *et al.*, 1993). It is possible, therefore, that during cooling a reduction in NO generation, through decreased NOS activity for example, would reduce negative feedback. A recent study showed that plasma cGMP levels did not rise in Raynaud's patients after cold-exposure, whilst those in control subjects were found to increase markedly (Leppert *et al.*, 1995), supporting the theory of a decreased production of endogenous NO during cooling. NOS activity may be decreased by a cold-induced conformational change or a reduction in calcium ion ($Ca^{2+}$) mobilisation from intracellular stores. The initial cold-induced decrease in NOS activity would be compensated for by an enhanced stimulation through ACh-muscarinic receptor binding. There is evidence to suggest that cooling increases the affinity of a wide range of agonists for their receptors. This may reflect changes in the fluidity of the cell membrane which reveal binding sites more easily on the receptor surface (Vanhoutte, 1980). In addition, increased formation of high affinity receptor-G-protein complexes, which are sensitive to temperature, have been shown to occur for a number of receptor types, including muscarinic (Aronstam & Narayanan, 1988), $\alpha_2$-adrenergic (Gantzos & Neubig, 1988), and calcitonin gene-related peptide (CGRP) receptors (van Rossum *et al.*, 1993). Studies using canine saphenous arteries and veins support such an increase in the affinity of muscarinic receptors during cooling, since both the constrictor (Vanhoutte & Shepherd, 1970) and dilator response (Soares de Moura & Vanhoutte, 1988) to ACh was found to be augmented by cooling.

Similarly at 37°C, but not at 24°C, endothelium-independent relaxation had a tendency to be reduced in arteries from Raynaud's patients compared to those from healthy controls. One might have expected to find an augmented response to SNP at 37°C in Raynaud's vessels if, as the ACh data would suggest, NO production is impaired owing to hypersensitivity of the vasular smooth muscle to exogenous NO (Shirasaki & Su, 1985; Moncada *et al.*, 1991). Perhaps then the response to ACh is not indicative of NO production, but instead reflects the generation of additional vasodilators, such as $PGI_2$ and EDHF, which have been shown to account for a greater proportion of relaxation than NO in the microvasculature (Nagao & Vanhoutte, 1993).

The results obtained using arteries from Raynaud's patients at 37°C are similar to those which have been reported in other conditions associated with vasospasm. Impaired relaxation to endothelium-dependent and -independent dilator substances was found in canine cerebral arteries following subarachnoid haemorrhage, despite a normal release of NO (Kim *et al.*, 1988; 1989). These arteries were subsequently shown to have a reduced activity of sGC (Kim *et al.*, 1992). This may also account for the depressed relaxation found in vessels from Raynaud's patients.

Since work began on this thesis, three clinical studies have examined dilator function in Raynaud's disease. The first of these compared the responsiveness of dorsal hand-veins to the endothelium-dependent vasodilator, bradykinin, and the endothelium-independent vasodilator, SNP, between Raynaud's patients and control subjects (Bedarida *et al.*, 1993). The results showed that endothelium-dependent venodilatation was impaired in patients with Raynaud's disease, whilst smooth muscle responsiveness to NO was similar in both groups. In a similar study, this time examining digital artery responses to acetylcholine (ACh), an endothelium-dependent vasodilator, and glyceryl trinitrate (GTN), an endothelium-independent vasodilator, Raynaud's patients were found to have a depressed dilator response to ACh compared

to controls (Singh *et al.*, 1995), again implying impaired vasodilatation exists in Raynaud's disease. In addition, dilatation to GTN was enhanced in subjects with Raynaud's, compared to controls. This finding supports the hypothesis of diminished NO-activity in Raynaud's disease, because, as mentioned earlier, it has been reported that the response to exogenous nitrovasodilators is potentiated after removal of NO-mediated vasodilator tone (Shirasaki & Su, 1985; Moncada *et al.*, 1991). The third study measured fingertip and forearm blood flow responses to brachial artery infusions of SNP and the endothelium-dependent vasodilator, methacholine (MCh) (Khan & Coffman, 1994). Interestingly, an enhanced response to MCh was found in Raynaud's patients compared to control subjects. No difference in the responses to SNP was shown. The authors were unable to suggest a mechanism responsible for these findings.

None of the above studies examined the relaxant response of the vasodilators during cooling. The results from the studies by Bedarida *et al.* (1993) and Singh *et al.* (1995) concerning endothelium-dependent dilatation are in agreement with the present data (Table 6.7), in that the cutaneous resistance arteries of patients with Raynaud's disease show impaired responsiveness to ACh at 37°C. Although the response to SNP was attenuated in vessels from Raynaud's patients compared to controls, the results were not statistically significant, and are comparable to those reported by Bedarida *et al.* (1993), who also found a non-significant decrease in the maximal dilatation. Singh and colleagues (1995) examined the relaxant response to a single dose of glyceryl trinitrate (GTN), so it is difficult to compare the present results with that study, but a diminished response to agonist-stimulated vasodilatation may reflect a diminished generation of dilator substances other than NO, in which case one would not expect to find a difference in smooth muscle responsiveness to NO.

***TABLE 6.7.*** *A comparison of the in vitro results presented in this thesis with published in vivo studies examining vasodilator responses in Raynaud's disease*

| | Endothelium - dependent | | Endothelium - independent | |
|---|---|---|---|---|
| | Agonist used | RD vs. control | Agonist used | RD vs. control |
| Bedarida *et al.*, 1993 | BK | ↓ | SNP | - |
| Khan & Coffman, 1994 | MCh | ↑ | SNP | - |
| Singh *et al.*, 1995 | ACh | ↓ | GTN | ↑ |
| Smith - Chapter 6 | ACh | ↓ | SNP | - |

*Bradykinin, BK; methacholine, MCh; acetylcholine, ACh; sodium nitroprusside, SNP; glyceryl trinitrate, GTN. ↓ / ↑ represent impaired/enhanced responses in Raynaud's patients compared to control subjects; - represents no significant difference of the responses between the two groups.*

Although conflicting, the results from the study by Khan & Coffman (1994) can perhaps be explained by the endothelium-dependent dilator used. Chowienczyk and colleagues (1993) have shown that the vasodilator response to MCh is unaffected by inhibitors of NOS, implying that MCh elicits dilatation through an NO-independent mechanism, which may account for the results from the study by Khan & Coffman (1994). In addition, a variability in the temperature of the subjects' vasculature may be responsible. It is unlikely that the blood vessels of the arm experience a temperature of 37°C; Bazett *et al.* (1948) demonstrated temperatures as low as 21.5°C for the radial and 31.1°C for the brachial artery in subjects who had thermocouples inserted into vessels in their arm. Therefore, the increased responsiveness to MCh may reflect an enhanced response to muscarinic receptor stimulation at temperatures below 37°C, possibly through increased receptor affinity which, as discussed earlier, has been shown to occur during cooling (Aronstam & Narayanan, 1988). When studying vascular responses *in vivo*, it is important to take into account possible temperature differences in the vasculature between subjects. Despite maintaining a constant room temperature, skin temperatures can vary significantly; Walmsley and Goodfield (1990) found that the Raynaud's patients in their study had a lower skin temperature

compared to controls. Perhaps the vessels under study in the patients used in the study by Khan & Coffman (1994) were at a lower temperature than those from the other two studies. This discussion raises some questions as to whether 37°C is an appropriate temperature to use in isolated vessel studies (see Chapter 7).

In the study by Bedarida *et al.* (1993) it was found that the Raynaud's patients who had antinuclear antibodies (ANA) present in their plasma, were among the lowest responders to bradykinin, although the sample size was insufficient to test statistical significance. There was a similar trend in the present study with respect to the responsiveness to ACh in vessels from Raynaud's patients, but again the sample size was too small to draw any firm conclusions.

The fact that a disorder of the vascular endothelium was observed in dorsal hand-veins of patients with Raynaud's disease (Bedarida *et al.*, 1993) provides further support for the use of arteries isolated from the gluteal region in this thesis. The study by Bedarida and co-workers demonstrates that endothelial dysfunction can be observed at sites beyond those which are directly affected, since Raynaud's disease is primarily an arterial condition.

# CHAPTER 7:
# GENERAL DISCUSSION

## 7.1. Summary

The results from the perfusion myograph studies of isolated rat mesenteric resistance arteries showed that the endothelium is an important modulator of the cold-induced effects associated with several vasoconstrictor agents, either by enhancing contraction through the release of vasoconstrictors such as endothelin-1 (ET) or thromboxane $A_2$ ($TXA_2$), or by depressing contraction through the release of dilators, such as nitric oxide (NO) or prostacyclin ($PGI_2$). Thus, the endothelium does not exert a uniform influence on the responsiveness of an artery to an agonist, but instead its modulatory effect is dependent on the individual agonist used.

The results from the studies of resistance arteries dissected from gluteal fat biopsies taken from control subjects and from patients with Raynaud's disease revealed that at 37°C arteries from Raynaud's patients showed an enhanced sensitivity to ET-1 in comparison with those from controls. A possible explanation for this could be increased release of endothelium-derived constrictors, as suggested by a reduction in sensitivity after endothelial removal, and/or a reduced dilator function in Raynaud's patients, as suggested by the responses to the endothelium-dependent dilator acetylcholine (ACh) and the endothelium-independent dilator sodium nitroprusside (SNP), which were both attenuated in vessels from patients with Raynaud's disease in comparison with controls. This implied that there was a decrease in smooth muscle sensitivity to NO, and possibly NO-synthase (NOS) activity, at 37°C. Interestingly, at 24°C, the responses to ET-1, ACh and SNP were similar in control arteries and in those from patients with Raynaud's disease. It would appear from the ACh data that NOS activity was enhanced in vessels from Raynaud's disease patients during cooling, with the increase in NO production opposing the contraction to ET-1. Nevertheless, this might only apply to agonist-stimulated NO production, which may be potentiated as a result of depressed basal production. The results obtained at 37°C support the hypothesis that vascular endothelial function is abnormal in Raynaud's disease. It is possible that cooling to 24°C does not represent the actual temperature

that the digital vasculature is subjected to, or at least not upon initial exposure to cold, and therefore the primary pathophysiological mechanism involved would be stimulated at a temperature above 24°C. The question then arises - what temperature is most relevant to the situation that occurs in digital arteries *in vivo*? It is unlikely that temperatures as high as 37°C are experienced according to Bazett and colleagues (1948), who reported that blood in vessels such as the radial artery may have a temperature between 20°C and 25°C. If universally true, this finding leads to re-interpretation of published *in vitro* data obtained from vessels at 37°C. The temperatures of 37°C and 24°C were chosen in the studies presented in this thesis because they are directly comparable with many published reports, and represent body temperature and moderate cooling. Ideally, one would measure accurately the temperature of the digital vasculature, under normal and cold conditions, and repeat the present studies at these temperatures in order to gain a closer insight into the mechanisms involved in cold-induced vasospasm. Ofcourse, the ultimate way of achieving physiological temperatures is to carry out *in vivo* studies in humans, but this has the disadvantage that not all drugs can be administered to human subjects.

The autoperfused hindlimb of the rat was set up as a model for studying mediators of cold-induced vasoconstriction, which may in turn reveal potential factors involved in the pathogenesis of Raynaud's disease. The use of a non-selective α-adrenoceptor antagonist supported a role for α-adrenoceptors in mediating cold-induced vasoconstriction, but it remains unclear whether this finding was indeed due to antagonism of α-adrenoceptors, since selective antagonists for $\alpha_1$- and $\alpha_2$-adrenoceptors were unable to attenuate the vasoconstriction induced by cooling in this model. There would appear to be a role for ET in mediating cold-induced vasoconstriction, perhaps through the $ET_A$-receptor, because there was a tendency for a selective $ET_A$-receptor antagonist to decrease the rise in hindlimb perfusion pressure during cooling - increasing the number of experiments may help to determine this role. Further studies, using inhibitors of the nitric oxide and prostacyclin pathways, could

be performed in order to examine the role of endothelium-derived vasodilators in cold-induced vasoconstriction *in vivo*.

Results obtained using the hindlimb preparation need careful interpretation, particularly in comparison with those from isolated blood vessels used in previous studies, with regard to the role of α-adrenoceptors in cold-induced vasoconstriction. However, there are advantages in using a functional preparation such as the autoperfused hindlimb, including: the innervation of the vascular bed is intact, a wide range of vessels are being studied, and blood-borne factors, including hormones, are present. A disadvantage of the model is the fact that the temperature-dependent changes in hindlimb perfusion pressure are the result of changes in blood flow to skeletal muscle rather than the cutaneous circulation; this may account for the apparent insensitivity to antagonist drugs. Perhaps a better preparation in which to investigate the effects of temperature on vascular reactivity would be the rat tail, which serves as an important thermoregulatory organ in this animal (Rand *et al.*, 1965). A recent study by Redfern and colleagues (1995) demonstrated the role of postjunctional $\alpha_2$-adrenoceptors in controlling blood flow, and hence heat loss, from the tail of the rat. This study highlights the similarities between the rat tail and the human digit, the arteries of which have been shown to possess postjunctional $\alpha_2$-adrenoceptors (Stevens & Moulds, 1986).

An important finding in this thesis is that the effects of temperature on the responsiveness of blood vessels cannot be predicted according to the type of vessel under study. As a general rule it has been said that cooling constricts cutaneous vessels and dilates other vascular beds (Vanhoutte, 1980). However, this does not necessarily imply that cooling potentiates the responses to contractile agents in cutaneous vessels, whilst inhibiting those non-cutaneous vessels. Many studies have found this not to be the case, including those in this thesis: contractile responses to ET-1 and phenylephrine were augmented in rat mesenteric resistance arteries *in vitro*,

and cooling was found to increase vascular resistance in the rat autoperfused hindlimb *in vivo*, a preparation which appears to reflect blood flow to the skeletal vascular bed. In his review of the effects of temperature on blood vessel function, Vanhoutte (1980) points out the difficulties in comparing data from different studies, even when using the same type of vessel. Many factors can influence the results, including time course, magnitude of temperature alterations, and acclimitisation, age or hormonal status of animal/subject preceeding the study.

## 7.2. Implications of present studies

When examining the results drawn from the present study, together with evidence from the literature, one can speculate on the mechanisms involved in the pathophysiology of Raynaud's disease. It appears likely that there is a dysfunction of the vascular endothelium in patients with Raynaud's disease, which causes a reduction in NO, $PGI_2$ and/or endothelium-derived hyperpolarising factor (EDHF) activity (Chapter 6; Bedarida *et al.*, 1993; Singh *et al.*, 1995). This primary dilator dysfunction will reduce negative feedback signals on ET-1 production, resulting in enhanced levels of circulating ET-1, as reported by Zamora and colleagues (1990). During cold exposure, or emotional stress, adrenaline is released from the adrenal medulla, and noradrenaline release (and co-release of neuropeptide Y and ATP) from adrenergic nerve terminals is increased through enhanced sympathetic nerve activity (Vanhoutte, 1980). Noradrenaline and adrenaline then act on $\alpha_1$- and $\alpha_2$-adrenoceptors to cause vasoconstriction. The present studies show that sub-pressor amounts of endogenous ET can potentiate the response to $\alpha_1$-adrenoceptor agonists (Chapter 4), confirming similar studies by Yang *et al.* (1990) in which exogenous ET-1 potentiated the response to noradrenaline and 5-hydroxytryptamine (5-HT). In the isolated rat tail, it has been demonstrated that the contractile response to $\alpha_2$-adrenoceptor agonists is dependent on the level of vascular tone present, whereby the contractile response is potentiated following the induction of tone (Templeton *et al.*, 1989; MacLean & McGrath, 1990). By extending this to the vasculature of Raynaud's

patients *in vivo*, the augmented contraction to $\alpha_1$-adrenoceptor stimulation may unmask $\alpha_2$-adrenoceptors present. In addition, many studies have shown that cooling potentiates contractile responses mediated by $\alpha_2$-adrenoceptors (e.g. Flavahan *et al.*, 1985). Vasospasm during cooling could therefore arise as a result of an increase in $\alpha_2$-adrenoceptor number and affinity, and $\alpha_1$-adrenoceptor-mediated release of ET. Because the endothelium in control subjects is functioning normally, ET production is constantly kept in check by opposing endothelium-derived vasodilators. Cold exposure or emotional stress will still increase sympathetic nerve activity, and augment vasoconstriction mediated by $\alpha_2$-adrenoceptors, but at the same time the affinity of $\alpha_2$-adrenoceptors present on endothelial cells will be increased. This will stimulate endothelium-derived dilator substances to be released and hence counteract the vasoconstriction. In control subjects, the overall balance favours vasoconstriction as a physiological response to cold or emotional stimuli. Vasospasm is prevented because NO, $PGI_2$ and EDHF are inhibiting ET-1 generation. In Raynaud's patients, however, the unopposed generation of ET is ultimately responsible for the observed prolonged vasospasm. The results from the dilator studies presented in Chapter 6 indicate that NOS activity, or indeed $PGI_2$ synthase, is markedly enhanced at 24°C. It makes sense that the arteries of Raynaud's patients have an increased NOS activity and/or $PGI_2$ synthase at 24°C; this temperature may represent a critical degree of cooling which activates mechanisms responsible for reversing the vasospasm in order to prevent damage to the digits and other affected extremities. A possible mechanism for inducing dilator function may be the loss of shear stress during a vasospastic attack. It is known that flow-induced shear stress regulates the production of $PGI_2$ (Frangos *et al.*, 1985) and NO (Rubanyi *et al.*, 1986). Perhaps then flow-induced $PGI_2$ and NO production is depressed during cold-induced vasospasm, causing the up-regulation of enzymes involved in the generation and/or action of the dilators. Stimulation of endothelial $\alpha_2$-adrenoceptors by increased levels of noradrenaline would result in an enhanced release

of vasodilator substances which would overcome the spasm, thus allowing the blood supply to return to the digits. This hypothesis is summarised in the diagram below (Figure 7.1).

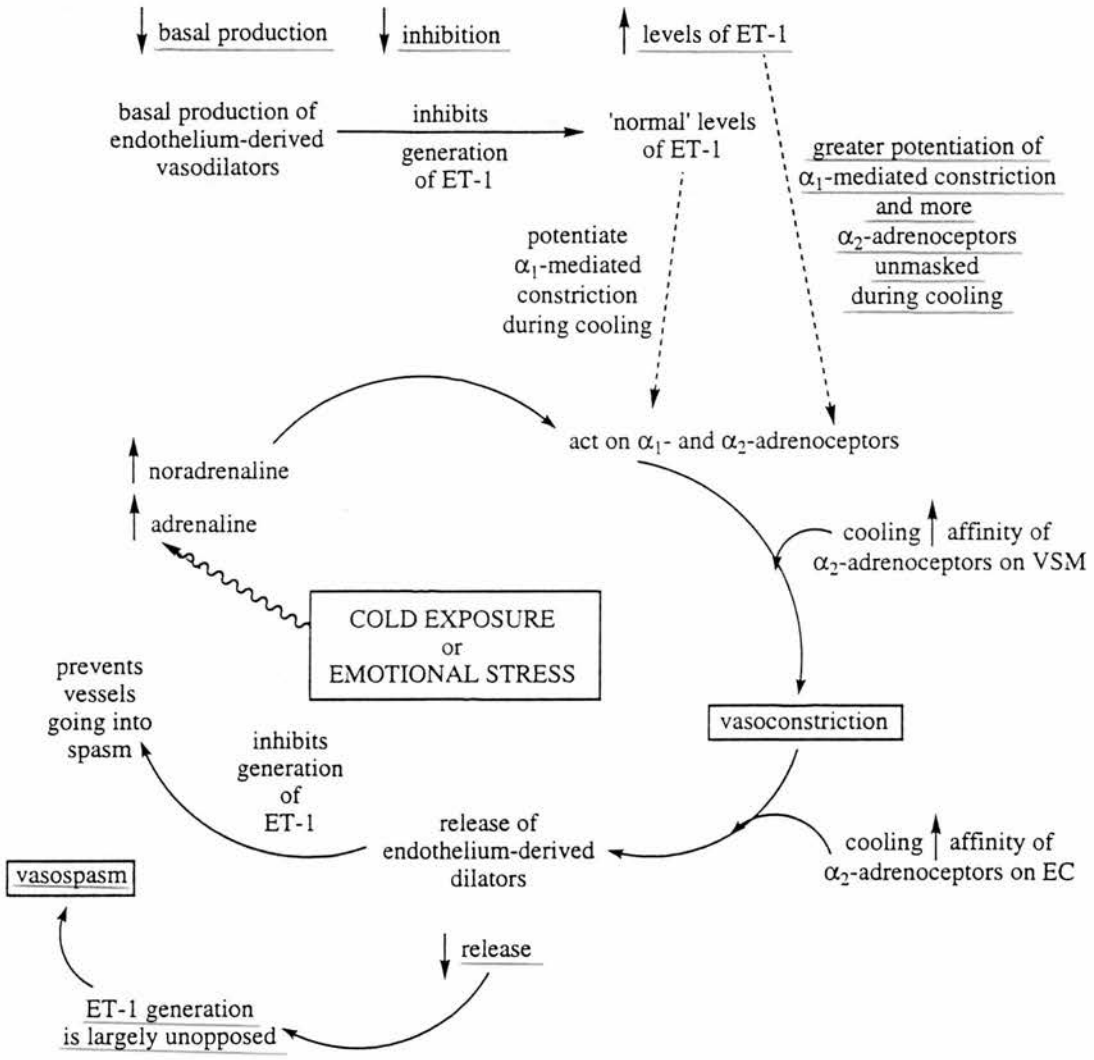


***Figure 7.1.*** *Schematic outlining the hypothesised pathway which may account for the vasospasm found in patients with Raynaud's disease. ET-1 = endothelin-1; VSM = vascular smooth muscle; EC = endothelial cells; ↓ = decrease; and ↑ = increase. Parts refering to Raynaud's patients are underlined in red.*

The hypothesis outlined above unites the theories of Maurice Raynaud and Sir Thomas Lewis, whereby overactivity of the sympathetic nervous system is attributed to potentiation of $\alpha_2$-adrenoceptors, and the 'local fault' resides in a dysfunction of endothelium-derived vasodilator substances.

## 7.3. Future directions

The most important results to come out of this thesis with regard to the pathogenesis of Raynaud's disease, are the studies in the isolated resistance arteries obtained from Raynaud's patients and control subjects. The observed differences between the rat and human data highlight the importance of studying vessels obtained from human subjects. Despite the difficulties in obtaining gluteal fat biopsies, the results from these studies are of greater potential significance to our understanding of cold-induced vasoconstriction and the pathophysiology of Raynaud's disease than vessels obtained from animal tissue. It is therefore vital to increase the numbers of biopsy studies in order to clarify, with statistical certainty, the pathogenetic role of the vascular endothelium in Raynaud's disease. Although the present results implicate a dysfunction of the endothelial-dependent dilator system, the involvement of ET is less clear. Hopefully, by increasing the number of vessels studied, the role of ET and NO in Raynaud's will be determined.

The involvement of the vascular endothelium in the pathogenesis of Raynaud's disease gives rise to new areas for drug development. Clinical trials have already shown beneficial effects of L-Arginine therapy, the substrate for NOS, in patients with secondary Raynaud's phenomenon (Agostoni *et al.*, 1991). If, in the light of further biopsy studies, ET does appear to have a role in the development of vasospasm, albeit due to a primary dysfunction of the endothelium-dependent dilator system, ET receptor antagonists should be examined as a potential treatment of Raynaud's disease. Orally active ET receptor antagonists, which have been shown to be effective in animal studies (Clozel *et al.*, 1993), are currently being developed for use in humans. It would be of interest to investigate the effectiveness of such drugs in preventing vasospastic attacks in Raynaud's patients. Appropriate studies could be carried out to determine if a selective $ET_A$ receptor antagonist would be preferable to a non-selective $ET_{A/B}$ receptor antagonist, such as bosentan, in Raynaud's disease. Vasospasm in Raynaud's disease is thought to occur primarily in the smaller arteries, so a non-

selective $ET_{A/B}$ receptor antagonist may be advantageous because $ET_B$ receptors have been shown to contribute to vasconstriction in human resistance arteries *in vitro* (Deng *et al.*, 1995) and *in vivo* (Haynes *et al.*, 1995). Blockade of these constrictor $ET_B$ receptors may outweigh the deleterious effects of blocking $ET_B$-mediated endothelium-dependent dilatation, which, as the results presented in Chapter 6 indicate, is impaired in any case in Raynaud's patients.

Preliminary macroautoradiographic studies which I have carried out in subcutaneous resistance arteries from control subjects reveal the presence of $ET_A$ and $ET_B$ receptors. Further quantitative autoradiographic studies could be carried out in vessels from patients with Raynaud's disease and healthy controls in order to determine whether there is a difference in the density and distribution of ET receptors. Perhaps Raynaud's patients have a reduced number of $ET_B$ receptors present on endothelial cells, or an enhanced number of $ET_A$ and $ET_B$ receptors on vascular smooth muscle cells, which would promote vasoconstriction.

The studies presented in this thesis suggest that endothelium-dependent dilator substances are affected by cooling. It remains unclear whether endogenous generation of these substances is diminished in Raynaud's patients, because agonist stimulated production may not reflect the situation *in vivo*. In order to investigate further the effect of cooling on the generation of NO, studies could be performed in cultured endothelial cells from Raynaud's patients and control subjects, in which NOS activity could be measured at 37°C versus 24°C, for example by a spectrophotometric assay, of the conversion of oxy- to methaemoglobin (Feelisch & Noack, 1987), or by a citrulline assay, of the conversion of [$^3$H]-L-arginine to [$^3$H]-L-citrulline (Salter *et al.*, 1991). The results from such studies would help to elucidate the mechanisms involved in the depressed endothelium-derived dilator function in control subjects, and the enhanced function in Raynaud's patients, during cooling.

The results from the hindlimb model are inconclusive regarding a role for ET in cold-induced vasoconstriction. Clearly further experiments need to be performed using ET receptor antagonists in an attempt to affirm the role of ET with confidence. However, as discussed in Section 7.1, a better model in which to study cold-induced vasoconstriction would be the rat tail, since this is a physiological thermoregulatory organ, and is perhaps more similar to the human digit. In addition, the role of endothelium-derived vasodilator substances could be explored by using inhibitors of the NO and $PGI_2$ pathways.

Since endothelial dysfunction has been implicated in the pathogenesis of Raynaud's disease and there are clear gender differences in the occurence of this disorder, it would appear likely that there is an interaction between endothelium-derived factors and sex hormones. It is well established that sex hormones alter the vascular responsiveness to various vasoactive factors, including α-adrenoceptor agonists (Vanhoutte *et al.*, 1981); less clear is their effect on the vascular endothelium. Recent evidence does support an interaction between female sex hormones and the endothelium (see Chapter 1, Section D.1.8). Progesterone (Williams *et al.*, 1988) and oestrogen (Gilligan *et al.*, 1994) have been shown to enhance endothelium-dependent dilatation . Future work, at the molecular level, could investigate the influence of sex hormones on the ET and NO pathways, for example on the activities of endothelin-converting enzyme (ECE) and NOS, or on the expression of ET receptors. Although serum levels of female sex hormones do not differ significantly between Raynaud's patients and controls (Bartelink *et al.*, 1994), it is feasible that women with Raynaud's disease are less responsive to progesterone and oestrogen, thus making them more prone to vasospasm.

## 7.4. Conclusions

The results presented in this thesis demonstrate that the vascular endothelium is an important modulator of cold-induced effects on the response to several vasoconstrictor

agents, either by enhancing contraction through the release of vasoconstrictors such as ET or $TXA_2$, or by depressing contraction through the release of dilators, such as NO or $PGI_2$.

At the outset of this thesis it was hypothesised that vasoconstriction to ET is opposed by increased NO production in healthy controls during cold-exposure, the balance favouring vasoconstriction (but not of sufficient severity or duration to cause vasospasm), and that patients with Raynaud's disease have a dysfunctional endothelium, perhaps resulting from an increased production of ET and/or reduced production of NO, favouring prolonged vasospasm. The results from the gluteal biopsy studies would largely support this hypothesis, in that vessels from Raynaud's patients displayed an enhanced sensitivity to ET-1 at 37°C, which was dependent on changes in dilator function, namely a depressed action of endothelium-derived vasodilators and smooth muscle sensitivity to NO. Although an increase in the response to ET-1 was not found at 24°C, the results obtained at 37°C would support the hypothesis that the release and/or actions of NO is reduced in Raynaud's disease, allowing enhanced constriction to ET. These results suggest, therefore, that the vascular endothelium is involved in the pathophysiology of Raynaud's disease. As indicated in Figure 7.1, α-adrenoceptors are also likely to play an important role in the development of cold-induced vasoconstriction and vasospasm.

These findings, together with the proposals for future work, indicate directions for the development of specific and effective treatment for Raynaud's disease, for which there is currently no generally effective therapy. In addition, since Raynaud's disease appears to be part of a more generalised vasospastic disorder, which includes variant angina and migraine, the present results may lead to a clearer understanding of the mechanisms underlying these other vasospastic conditions, and again point to new directions for drug development.

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